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CALCIUM-MEDIATED CHANGE IN NEURONAL INTRINSIC EXCITABILITY IN WEAKLY ELECTRIC FISH: BIASING MECHANISMS OF HOMEOSTASIS FOR THOSE OF PLASTICITY

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by

Andrew A. George, B.S.

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Dedication

This dissertation is dedicated to my wife Linda L. George. Her faith, love and unwavering patience have given me courage through times of radiant creativity and arduous change. Her strength has become my strength and I can only hope and pray that all who value scientific investigation have such a kind and loving companion.

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Finally, a very special thanks is reserved for my wife, Linda L. George. Linda has been my inspiration and my hope for better days to come. Her kindness and overwhelming encouragement over the years have helped me cope with the many difficult challenges I faced in graduate school. She helped me make rational decisions when I was completely and utterly irrational. She is a person of high distinction and I am grateful that we made this journey together.

CALCIUM-MEDIATED CHANGE IN NEURONAL INTRINSIC EXCITABILITY IN WEAKLY ELECTRIC FISH: BIASING MECHANISMS OF HOMEOSTASIS FOR THOSE OF PLASTICITY

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Although the processes used for temporarily storing and manipulating neural information have been extensively studied at the synaptic level far less attention has been given to the underlying cellular and molecular mechanisms that contribute to change in the intrinsic excitability of neurons. More importantly, how do these mechanisms of plasticity integrate with ongoing mechanisms of regulation of neural intrinsic excitability and, in turn, homeostasis of entire neural circuits?

In this dissertation I describe the underlying mechanisms that contribute to persistent neural activity and, more globally, sensorimotor adaptation using weakly electric fish as my model system. Weakly electric fish have evolved a behavior

adaptation known as the jamming avoidance response (JAR), and it is this adaptation that allows the organism to elevate its own electrical discharge in response to intraspecific interactions and subsequent distortions of the animal's electric field. The elevation operates over a wide range and *in vivo* can last tens of hours upon cessation of a jamming stimulus.

I demonstrate that the underlying mechanisms of the adaptation are mediated by calcium-dependent signaling in the pacemaker nucleus and that calcium-mediated phosphorylation plays an important role in the regulation of the long-term frequency elevation (LTFE). I demonstrate using an *in vitro* brain slice preparation from the weakly electric fish, *Apteronotus leptorhynchus* that the engram of memory formation depends on the cooperativity of calcium-dependent protein kinases and protein phosphatases.

In addition, I show that the memory formation (in the form of LTFE) does not depend on the continued flux of calcium, but rather the phosphorylation events downstream of NMDA receptor activation. Moreover, I describe the differences in the expression of protein phosphatases and protein kinases as they relate to species-specific differences in sensorimotor adaptation. It is important to note that this is the first time that the cooperativity between different isoforms of protein kinase C (PKC) have been shown to play a role in graded long-term change in neuronal activity and, in turn, providing the neural basis of species-specific behavior. The neural adaptation of the electromotor system in weakly electric fish provides an excellent model system to study the underlying cellular and molecular events of vertebrate memory formation.

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Chapter 1: Introduction

NEURONAL HOMEOSTASIS

The behavior of neural networks depends on the complex interaction between the dynamics of the individual neurons (i.e. their intrinsic properties that are based on the numbers and types of voltage-gated ion channels in their membranes) and their synaptic strengths (Marder et al., 1996). For many years, changes in synaptic efficacy were the main focus in explaining the plastic changes in neural dynamics or what is commonly referred to as memory. Experimental work that has spanned over two decades, using various models from the invertebrate gill-withdrawal reflex in *Aplysia* to long-term potentiation (LTP) in the mammalian hippocampus, have demonstrated that long-lasting changes in synaptic strength could be produced. The mechanisms underlying those changes could be identified as those that regulate stability and plasticity in neural circuits and, ultimately, generate memory formation.

Homeostasis is a complex set of mechanisms that ensures constancy in the internal cellular environment. Neurons posses the homeostatic mechanisms that are necessary to ensure stable function over an animal's lifetime (Marder and Prinz, 2003). To understand how these homeostatic mechanisms might function, it is imperative to understand how synaptic and intrinsic properties must be adequately regulated for precise network performance and, coupled with compensatory mechanisms, allow for multiple solutions to reproduce the same behavior (Marder and Goaillard, 2006).

Most studies of long-term changes in synaptic strength have concentrated on Hebbian mechanisms, where an increase in synaptic efficacy arises from the presynaptic cell's repeated stimulation of the postsynaptic cell (Bloodgood and Sabatini, 2007; Laezza et al., 1999; Leslie et al., 2001). The Hebbian mechanisms that underlie plasticity

are crucial for modifying neuronal circuitry selectively, but have the potential to drive neural activity toward runaway excitation or quiescence (Turrigiano, 1999). Recently, several forms of homeostatic plasticity that stabilize the properties of neural circuits have been identified (Gong et al., 2007; Shah and Crair, 2008; Wierenga et al., 2005). Suggesting that neurons have a set point of activity and can be maintained by several mechanisms including synaptic scaling, regulation of neural intrinsic excitability, activity dependent receptor trafficking and activity dependant regulation of synapse number (Wallace and Bear, 2004; Wang et al., 2002; Wilhelm et al., 2009; Xu et al., 2005).

The first, synaptic scaling, suggests that the strength of all neuronal properties can be shifted up or down its input/output curve **(Figure 1A)**. This determines how fast the neuron fires for a given amount of synaptic drive. In contrast, the regulation of intrinsic conductances, or intrinsic plasticity, states that a neuron can modify the input/output curve of the neuron by shifting the curve left or right, essentially maintaining the output value (i.e. activity of the neuron) despite the changes in synaptic input **(Figure 1B)**. In cultured neocortical pyramidal neurons prolonged activity blockade lowers the threshold for spike generation, and neurons fire at a higher frequency for any given level of current injection (Desai et al., 1999).

The intrinsic excitability of neurons could potentially serve as a target for synaptically driven signaling cascades leading to long-term memory (Aizenman and Linden, 2000). These changes are manifest as changes in the structure and function of voltage-dependent ion channels expressed within a cell. In addition, synaptically driven changes could alter the signaling mechanisms that regulate ion channel kinetics and ultimately the homeostasis of entire neural circuits (Allen et al., 2000; Sytnyk et al., 2006; Thiagarajan et al., 2002). However, the plasticity would have to be constrained within the natural behavior that is relevant to the organism (Kobayashi et al., 2005;

Moosmang et al., 2005). Network function can be modulated to maintain this set point, including the strength of excitatory and inhibitory connections, and the intrinsic excitability of individual neurons. This occurs through modifications in the magnitude of voltage-dependent sodium current (a net overall increase), and a decrease in persistent potassium currents (Turrigiano, 2008; Turrigiano and Nelson, 2000). In light of these modifications, the exact mechanism that determines neuronal sensitivity is still unclear. A neuron may average its firing rate, or calcium concentration locally and/or globally (Golomb et al., 1994; Leslie et al., 2001; Vanderklish and Edelman, 2002).

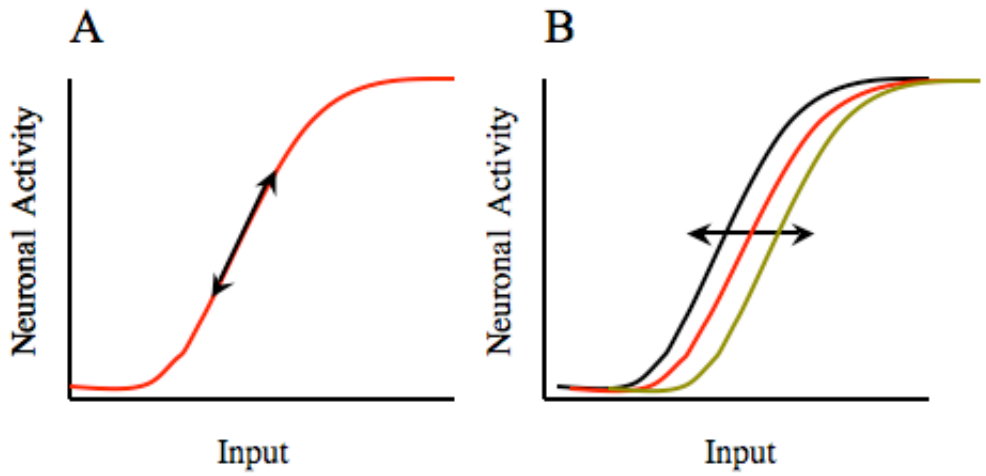


Figure 1: Models of homeostasis in neuronal plasticity

Homeostatic plasticity uses some measure of activity to adjust excitatory and inhibitory synaptic strengths, as well as the voltage-dependent conductances (Na+ and K+) that control neuronal firing properties. Model of synaptic scaling (Panel A) where a neuron's properties can be shifted up or down its input/output curve. The excitatory and inhibitory inputs can be regulated independently allowing neurons to adjust the balance of excitation and inhibition. Regulation of intrinsic conductances (Panel B; 'intrinsic plasticity') can modify the input/output curve of the neuron, by shifting it left or right (so it will fire the same for a given level of synaptic drive). Intrinsic plasticity changes the sensitivity of a neuron to both excitatory and inhibitory inputs, maximizing the detection of any input (modified from Turrigianno and Nelson, 2000).

Synaptic mechanisms for storing and manipulating information have been extensively studied (Kapur et al., 1997; Seamans et al., 2003; Wang et al., 2008; Yoshida et al., 2008), with less attention devoted to the underlying cellular mechanisms that restore normal patterns of activity when destabilizing effects, such as transient synaptic stimulation, perturb network activity. This raises the question of how ongoing postsynaptic mechanisms that regulate neural intrinsic excitability integrate synaptic input to produce stable, long lasting changes in neural output.

If the excitability depends on ion channels, then the question arises: "what processes govern the activity, expression, and distribution of ion channels in the membrane so that each neuron can maintain its electrophysiological identity?" With remarkable precision, neurons are able to fire appropriately in response to inputs over significantly different timescales (Pawlak et al., 2005). They must regulate the activity of ion channels expressed while synthesizing and inserting channels under potentiating events (Watt et al., 2000). Several models suggest that the excitability of a cell, as determined by increases in intracellular Ca^{2+}, regulates the maximal conductances of each of the ionic currents expressed in a neuron (LeMasson et al., 1993; Marder et al., 1996).

CALCIUM'S CONTRIBUTION TO NEURAL PLASTICITY

Ca^{2+} plays an important role in regulating a great variety of neuronal processes from triggering neurotransmitter release to mediating excitability, synaptic plasticity, and gene transcription (Neher and Sakaba, 2008; Park et al., 2008; Young et al., 2005). The intracellular Ca^{2+} concentration has important roles in the triggering of neurotransmitter release and the regulation of short-term plasticity (Oswald et al., 2006). In nerve terminals, Ca^{2+}-triggering of neurotransmitter release is achieved by activating a low-affinity Ca^{2+} sensor and requires high Ca^{2+} concentrations. The Ca^{2+} signal is transient

and is constrained to the vicinity of open Ca^{2+} channels (Neher and Sakaba, 2008). At the postsynaptic level, the activation of *N*-methyl-*D*-aspartate receptors (NMDARs), a non-selective cation channel, leads to an elevation in internal Ca^{2+} that can trigger distinct signaling mechanisms and, ultimately, enhance synaptic function (Malgaroli et al., 1992). The quintessential example of this enhanced function is demonstrated by long-term potentiation in the Schaffer Collateral pathway in the Hippocampus. High frequency stimulation of presynaptic axons can activate postsynaptic NMDA receptors and trigger the influx of calcium when glutamate binds to the postsynaptic NMDAR and the activation of AMPA receptors (Alpha-amino-3-hodroxy-5-methylisoxasole-4-proprionic acid) causes sufficient depolarization of the postsynaptic cell to expel Mg^{2+} from the pore of the NMDAR. When Mg^{2+} is expelled, Ca^{2+} can influx into the postsynaptic cell, initiating the persistent enhancement of synaptic transmission by acting on a number of intracellular effectors that bind calcium directly or through adaptor complexes.

NMDARs act as a coincidence detection mechanism in neurons whereby pairing of an action potential with synaptic stimulation results in a supralinear calcium accumulation within the spine (Yuste et al., 1994). Ca^{2+} supralinearity is defined as calcium transients with a magnitude greater than the sum of its component parts (i.e. it is larger than the computed sum of the calcium influx arising from both the action potential and synaptic activation). This has been demonstrated in backpropagating action potentials where synaptic activation results in the generation of robust long-term potentiation (LTP; (Magee et al., 1995). However, neurons possess firing levels that vary with the stimulus parameters in a graded manner and differences in intracellular calcium concentrations can account for such variability (Loewenstein and Sompolinsky, 2003).

The nonlinear and local nature of calcium dynamics enables neurons to generate a wide repertoire of spatiotemporal patterns of calcium levels, which are used to control

processes such as cell proliferation and development, but also cell death through necrosis and apoptosis (Chan and Mattson, 1999; Howard et al., 2003). In neurons, Ca^{2+} modulates membrane potential, synaptic release, short- and long-term plasticity and axonal growth (Becker et al., 1998; Gomis-Ruth et al., 2008). Graded persistent activity relies on activity-dependent $[Ca^{2+}]_i$ changes and has been found in single neurons in slices of entorhinal cortex (Egorov et al., 2002; Frank and Brown, 2003; Fransen et al., 2006). This area is known to be associated with memory functions, and the established persistent activity has been proposed as a single-neuron mechanism underlying working memory (Fransen et al., 2006).

These phenomena can result from regenerative Ca^{2+} signaling that involves the release of Ca^{2+} from internal stores into the cytoplasm. Various channels whose principal activator is the cytoplasmic Ca^{2+} concentration itself gate this release. Increasing intracellular Ca^{2+} facilitates further release of calcium from the stores, a positive-feedback cycle known as calcium-induced calcium release (CICR) from the endoplasmic reticulum (ER) via ryanodine receptors (or IP3 receptors). Interestingly, Ca^{2+} release has been shown to be regulated by calcium-mediated enzymes such as protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II, CaMKII (Kang and Othmer, 2007; Larabell et al., 2004; Lisman and Goldring, 1988; Oestreich et al., 2009).

CALCIUM-MEDIATED NEURAL SIGNALING AND PLASTICITY

A critical role of Ca^{2+} in neuronal signaling is to couple electrical excitation to the activation of intracellular enzymes and signal transduction cascades. Previous studies have demonstrated that learning events induce the activation of kinases with various spatial and temporal dynamics (Dash et al., 2007; Lisman et al., 2002; Lisman and

Zhabotinsky, 2001; Shobe, 2002). Phosphorylation and dephosphorylation of structural and regulatory proteins are major intracellular control mechanisms in eukaryotes.

Protein phosphorylation is the most common form of protein modification within the central nervous system and can use Ca^{2+}-dependent enzymes. Phosphorylation and dephosphorylation of structural and regulatory proteins are major intracellular control mechanisms in eukaryotes (Mizunuma et al., 2005). Protein kinases transfer a phosphate from ATP to a specific protein to serine, threonine, or tyrosine residues. Phosphatases remove the phosphoryl group and restore the protein to its original dephosphorylated state. Hence, the phosphorylation-dephosphorylation cycle can be regarded as a molecular "on-off" switch (Fox and Heitman, 2002; Groth et al., 2003; Nakajima-Shimada et al., 2000).

Protein kinases can be divided into two classes, those that are regulated by known second messengers (e.g. cAMP, cGMP, and Ca^{2+}) and those that are not (the Ca^{2+} - and cyclic nucleotide-independent protein kinases). The cyclic-nucleotide dependent protein kinases can be subdivided into two classes: 1) cyclic AMP-dependent protein kinases and 2) cyclic GMP-dependent protein kinase. Calcium-dependent protein kinases can be divided into 3 distinct subclasses 1) Ca^{2+}/calmodulin-dependent protein kinases, 2) myosin light chain kinase and phosphorylase kinase and 3) Ca^{2+}/phospholipid-dependent protein kinases. Calcium and cyclic nucleotide-independent protein kinases are classed into 3 distinct categories: 1) serine/threonine specific 2) tyrosine-specific protein kinases and 3) miscellaneous regulated protein kinases. The regulation of the state of phosphorylation of specific substrates by a variety of protein kinases appears to be a general mechanism by which many hormones, neurotransmitters, and other extracellular signals produce their physiological responses in specific target neurons (Nairn et al., 1985).

Recent evidence suggests that protein kinase C (PKC) contributes to enhanced synaptic efficacy at hippocampal CA1 synapses by mediating NMDAR-dependent long term pontentiation (Lan et al., 2001). In addition, an increase in postsynaptic PKC activity enhances synaptic transmission and, conversely, inhibition of PKC activity by intracellular delivery of PKC inhibitors blocks induction of LTP (Hu et al., 1987; Malinow et al., 1989).

To date, 11 protein kinase C isoforms have been identified and classified into three groups based on their structure and regulation **(Figure 2A)**. The best characterized and first discovered are the conventional PKCs. This group consists of PKCα, two alternatively spliced variants βI and βII, and γ. These isoforms are regulated by Ca^{2+} and diacylglycerol, (DAG). Additionally, the novel PKCs: δ, ε, η, υ, and μ isoforms are structurally similar to the conventional PKCs with the exception of the c-terminal activation and regulatory domain. The novel PKCs do not possess the functional motifs that mediate Ca^{2+} binding. Lastly, and least understood, are the atypical PKCs: ζ and ι/λ. Atypical PKCs differ significantly in structure from the other two classes. These isoforms have been reported not to respond to phorbol esters (phorbol 12-myristate 13-acetate (PMA); activators of conventional and novel PKCs that bind competitively to the DAG binding site) *in vivo* or *in vitro* and are regulated by protein-protein interactions and by direct phosphorylation by PDK1.

Protein phosphatases (PPs) have been classified into three distinct categories: 1) serine/threonine specific (Ser/Thr), 2) tyrosine-specific, and 3) dual-specificity phosphatases (the last two classes will not be discussed in this chapter). Based on biochemical parameters and substrate specificity, Ser/Thr protein phosphatases are divided into two major classes. Type I phosphatases, which include PP1, and Type II phosphatases which are subdivided into spontaneously active (PP2A), Ca^{2+}- dependent

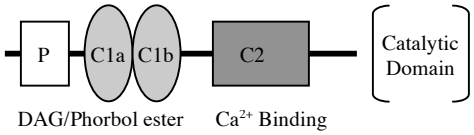
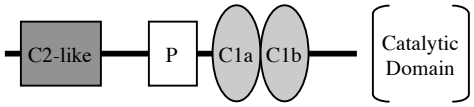
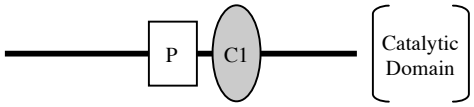
(PP2B), and Mg^{2+}-dependent (PP2C) classes of phosphatases (Selke et al., 1998). Over the past several decades, research has demonstrated that Ca^{2+} and calcium-mediated enzymes are critical regulators of a diverse array of ion channels, leading to both short and long-term effects on neuronal excitability and function (Soderling, 2000).

In LTD, the role of phosphatases differs from that of kinases in that the amount of calcium entry determines the activation of these effectors. In one form of LTD, activation of postsynaptic NMDA receptors with low frequency stimulation causes a relatively low local change in postsynaptic Ca^{2+} concentration and, in turn, leads to the activation postsynaptic PP1 or PP2A. The induction of LTD is blocked by the extracellular application of either okadaic acid or calyculin A, two inhibitors of PPl and PP2A (Mulkey et al., 1993).

Calcineurin (PP2B) is a eukaryotic Ca^{2+} and calmodulin-dependent serine/threonine protein phosphatase. It is a heterodimeric protein consisting of a catalytic subunit calcineurin A, which contains an active site dinuclear metal center, and a tightly associated, myristoylated, Ca^{2+}-binding subunit calcineurin B (**Figure 2B**). The primary sequence of both subunits and heterodimeric quaternary structure are highly conserved from yeast to mammals. As a serine/threonine protein phosphatase, calcineurin participates in a number of cellular processes and Ca^{2+}-dependent signal transduction pathways (Ruznak and Mertz, 2000).

Cyclosporin A and FK506 (specific calcineurin antagonists) are able to block the induction of LTD in the mammalian visual cortex and hippocampus (Hodgkiss and Kelly, 1995; Torii et al., 1995). Thus, LTD induction may require initial activation of CaN by Ca^{2+}/calmodulin. Calcineurin can dephosphorylate and inactivate inhibitor-1 (also known as DARPP-32), and this in turn increases PP1 activity and contributes to the generation of LTD (Malenka, 1994; Mulkey et al., 1994; Hodgkiss and Kelly, 1995b).

A

Subgroup	Isoforms	Activators	Regulatory Domain Structure
Conventional PKCs	α,βI,βII,γ	PS, Ca^{2+}, DAG, Phorbol ester	
Novel PKCs	δ, ε,μ,η,θ	PS, DAG, Phorbol ester	
Atypical PKCs	ζ, ι/λ	PIP_3, PS, Ceramides	

B

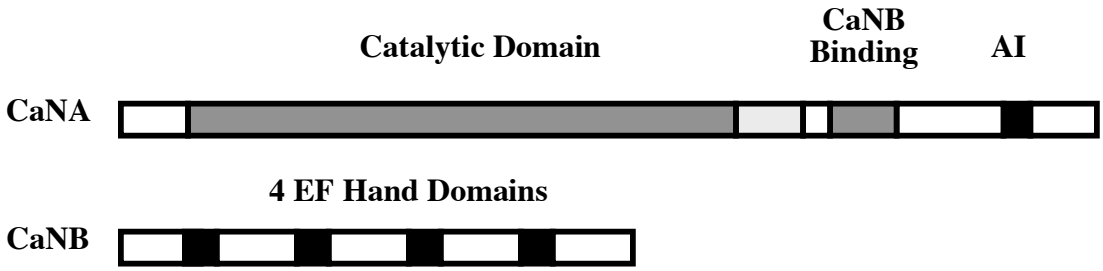


Figure 2: Activators and regulatory structures of PKC isoforms and calcineurin

(A) Conventional, Novel, and Atypical PKCs constitute the 3 main subgroups of the PKC family with each subgroup containing specific isoforms and modes of activation. PS = phosphatydilserine; DAG = diacylglycerol; PIP3 = phosphatidylinositol-3,4,5-triphosphate; P = pseudosubstrate. (modified from Azzi et. al, 1992). (B) Calcineurin A subunit (CaNA) has specific domains: a catalytic domain, a calcineurin B (CaNB)-binding domain, a calmodulin (CaM)-binding domain, and an autoinhibitory domain (AI). A regulatory subunit CaNB, which has 4 EF-hands that bind to Ca^{2+}, has a structure similar to CaM. B: In the regulation of CaN, an increase of intracellular Ca^{2+} triggers the binding of CaM to a CaNA/CaNB heterodimer and dissociates the carboxy-terminal AI domain from the catalytic groove. This structural change activates CaNA and is reversible. On the other hand, the drug-immunophilin complexes such as CsA-cyclophilin A and FK506-FKBP bind to a CaNA/CaNB heterodimer through the latch domain of CaNB and inhibit CaN activity competitively by covering the catalytic groove.

Chapter 2: Electroreception

ELECTRORECEPTIVE FISH

Freshwater weakly electric fish have evolved novel mechanisms of signal generation and detection independently in two geographically distinct groups: The Mormyriformes (Osteoglossomorpha, Teleostei) from Africa and the Gymnotiformes (Ostariophysi, Teleostei) from South America (Kramer, 1996). Both mormyriforms and gynmotiforms have evolved species that generate electric fields around their bodies through a specialized electric organ and utilize the electric organ discharge (EOD) for electrolocation and electrocommunication (Hopkins et al., 1997). The electric organ discharge (EOD) is gender and species-specific and can vary in frequency, waveform, amplitude, interpulse interval, and discharge pattern (Hopkins, 1988). A fish can sense EODs of conspecifics (through specialized sensory receptors known as electroreceptors) either by certain interference patterns or on the basis of the EOD alone (Heiligenberg, 1990).

Despite the phylogenetic distance between these two groups, weakly electric fish have evolved electrosensory perception primarily on the basis of relevant ecological and environmental constraints (Alves-Gomes et al., 1995). The EOD should reflect the best compromise between the generation of a signal that maximally exploits the sensitivity of the electroreceptors allowing for efficient electroreception and communication while at same time remaining cryptic to predators (Bradbury and Vehrencamp, 1998). In addition, the differential physical properties of the animal's environment (i.e. the capacitive or resistive properties of differential environmental elements) cause variation in the spectral components of the EOD (von der Emde, 1999).

However, electric organs do not represent an ancestral vertebrate trait but rather have evolved as a complement to perceive electrical signals and are sensed by specialized sensory receptors known as electroreceptors (Zakon, 1986). Electroreceptors are sensory cells derived from hair cells, forming part of the octavo-lateral sensory system and convey mechanoreceptive sensory information including the maintenance of equilibrium, the detection of gravity and rotation, and of water currents along the body (Bullock, 1973; Zakon et al., 1998). Weakly electric fish express two main types of specialized electroreceptors on the surface of their skin (Suga, 1967; Szabo, 1965), 1) Ampullary receptors, responding only to DC and low-frequency electric fields (e.g. < 50 Hz) and 2) tuberous electroreceptors, which are finely tuned and activated by a fish's own electric organ discharge (Zakon, 1986).

Ampullary electroreceptors are sensitive to weak electric field gradients 1-5 μV/cm in freshwater fishes (Kalmijn, 1988; Peters and van Ieperen, 1989; Zakon et al., 1998; Zakon, 1986) As stated previously, ampullary receptors respond to D.C. or low-frequency stimuli (~ 0.5 to 50 Hz) only. For weak stimuli, primitive ampullary receptors, such as those found in cartilaginous fishes, nonteleost bony fishes and amphibians, respond best to an externally negative stimulus, underlining their common origin (Gibbs, 2004). This is in contrast to the few teleost taxa that possess ampullary receptors responding best to (weak) stimuli of the opposite polarity (Zakon et al., 1998).

Tuberous electroreceptors are found only in a few teleost including the electrogenic Mormyriforms and Gymnotiformes (Bennett and Clusin, 1979; Srivastava and Szabo, 1974; Zakon et al., 1998; Zakon, 1986). Tuberous electroreceptor organs are activated by electric organ discharges (EODs). They are comprised of two functionally and morphologically distinct types: 1) the rapid timing class or T-type electroreceptor that follow each EOD cycle (i.e. time marker units of high sensitivity and short, fixed

latency to a supra-threshold EOD) and 2) the amplitude-modulated class or P-type electroreceptor whose probability of activation depends on changes in EOD amplitude. Tuberous electroreceptors tend to have bandpass characteristics (i.e. the ability to tune to a certain "best" frequency (Zakon 1986). This enables weakly electric fish to detect the presence of objects in their self-generated electric field (electrolocation; **Figure 3**), and secondly, detect the species- and gender-specific electric organ discharges generated by other conspecifics (i.e. electrocommunication).

ELECTROCOMMUNCATION

Communication signals in vertebrates must be **detectable**, **recognizable** and **memorable** (Guilford and Dawkins, 1993). The detectability depends on the filtering properties of electroreceptors (Zakon 1988), the recognition of a communication signal relies heavily on the faithful transmission of the discharge by tuberous electroreceptors and computation through higher neuronal mechanisms (Bell, 1989), and the recollection and/or memory of communication signals relies on underlying cellular and molecular mechanisms that contribute to the consolidation of information in higher neural processing centers.

Besides passive electrolocation, weakly electric fish use electric signals in active electrocommunication. They can display a wide variety of EOD modulations in different social contexts (Bullock, 1982; Heiligenberg and Rose, 1986; Hopkins, 1988). Two species of gymnotiform weakly electric fish, *Apteronotus leptorhynchus* and *Eigenmannia virescens,* exhibit differences in social structure (Oestreich and Zakon, 2005) and have developed a repertoire of behavioral adaptations and communicatory modifications (**Figure 4**).

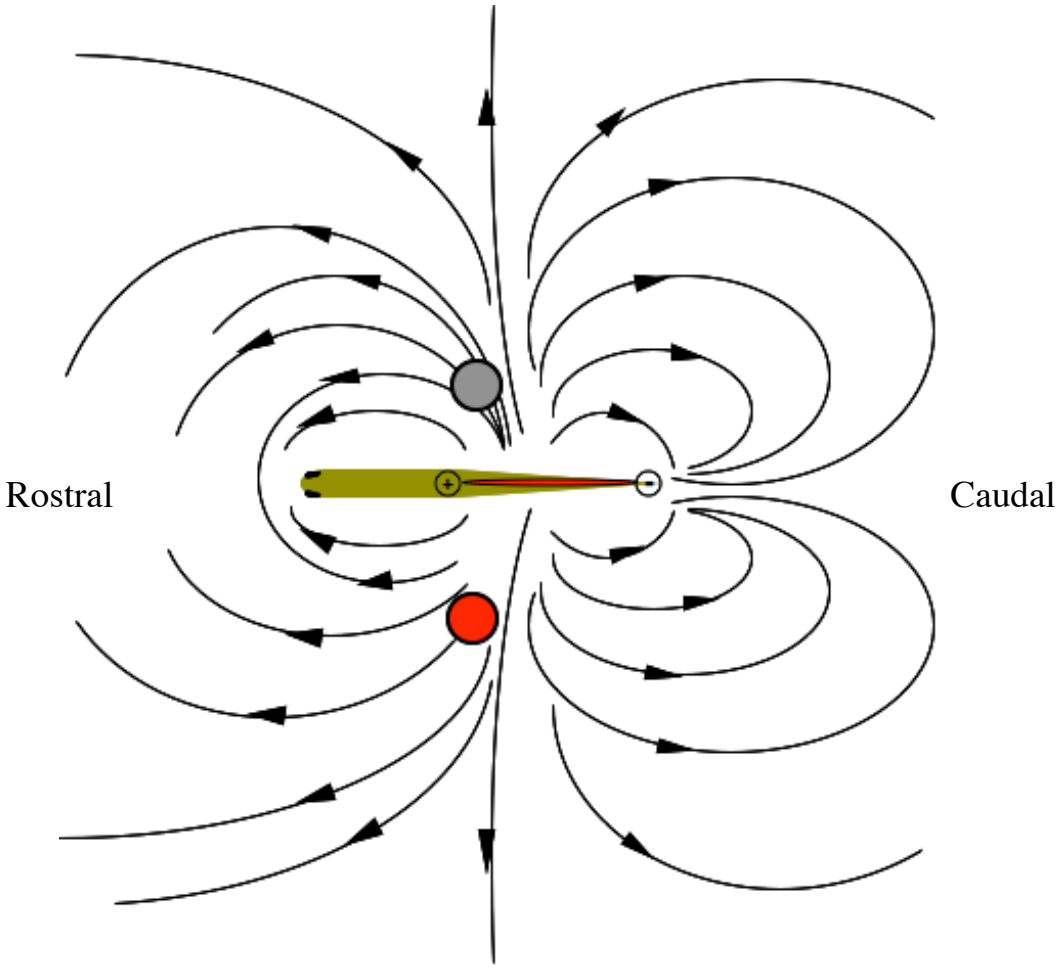


Figure 3: Schematic representation of active electrolocation in weakly electric fish.

The electric organ discharge (EOD) is produced by an electric organ in the tail of the fish (indicated by the red oval). The field distortions caused by objects of a conductivity different from that of the surrounding water are detected by the fish as a change in the voltage gradient in the area of the skin next to the object. Good conductors (as indicated by the gray circle) increase the transepidermal voltage gradient for local electroreceptors while insulators (indicated by the red circle) have a decreasing effect (modified from (Heiligenberg, 1977; Scheich, 1974).

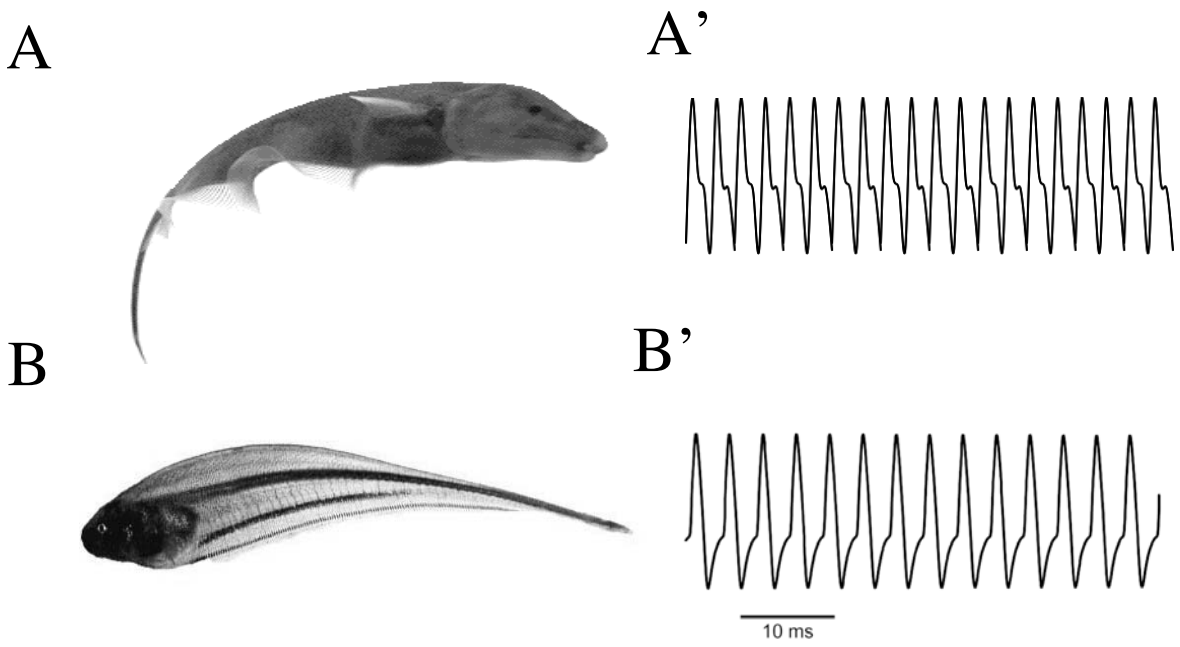


Figure 4: Gymnotiform weakly electric fish.

The brown ghost knife fish, *Apteronotus leptorhynchus* with its species-specific EOD (Panels A and A') and the glass knife fish, *Eigenmannia virescens* with its species-specific EOD (Panels B and B'). Modified from Harvey-Girard, 2005 and Carlson, 2006.

Apteronotus and *Eigenmannia* can be induced to modulate their EODs in several characteristic ways that are simple and stereotyped (Bullock, 1970; Hagedorn and Heiligenberg, 1985; Hagedorn et al., 1988; Hopkins, 1974). One behavioral modification used in courtship and agonistic displays is known as a chirp. Chirps are brief and rapid accelerations in the normally highly regular electric organ discharge (EOD) frequency (Dye, 1987). 'Chirping' or 'pinging' of the *Apteronotid* EOD was first described as a brief and spontaneous acceleration in frequency (Larimer and MacDonald, 1968). Chirping appears during experimental presentation of an artificial sine wave (mimicking conspecifics) and is usually interpreted as an aggressive signal (Hagedorn and Heiligenberg, 1985).

In addition to chirping, a non-selective, gradual rise in EOD frequency, known as a yodel, occurs after cessation of a stimulus and is clearly distinguishable from chirping behavior (Dye, 1987). Yodels appear to have evolved as 'victory-cries', an effect characterized as a signal to a retreating conspecific, or perhaps spontaneous discharge of lingering excitation (an anxiety release; Dye 1987).

THE JAMMING AVOIDANCE RESPONSE

The third, and perhaps the most intriguing behavioral adaptation, is known as the jamming avoidance response (JAR). *Apteronotus* discharges a quasi-sinusoidal electric organ discharge (EOD) in a species-specific range of frequencies of 600 to 1000 Hz. The EOD is sexually dimorphic, with mature males typically having discharge frequencies of 800 to 1000 Hz and females having discharge frequencies of 600 to 800 Hz. If two fish with similar EOD frequencies come in close proximity the interaction of both EOD signals interferes with the animal's electrosensory system, and disturbs its ability to effectively electrolocate objects and other organisms in its environment.

A fish will prevent jamming by transiently raising its own discharge frequency to a new, stable frequency and maintain the new frequency elevation for the entire period of the interaction (**Figure 5A**). In a series of well-described experiments, it was shown that the JAR can also be evoked experimentally by presenting the fish with an artificial sine wave signal of similar frequency (Oestreich and Zakon, 2002). The JAR is generated by the recruitment of normally quiescent, descending inputs from a higher-order nucleus (the sublemniscal prepacemaker nucleus; SPPn) that activates *N*-methyl-*D*-aspartate receptors (NMDARs) expressed in pacemaker and relay neurons (Harvey-Girard et al., 2007; Metzner, 1993).

The usual paradigm used to study the JAR was to present fish with a stimulus of up to 2 min, after which its EOD frequency returns to its baseline value (Bullock et al., 1972a; Dye, 1987; Dulka and Maler, 1994; Heiligenberg et al., 1996; Takizawa et al., 1999). More recent experiments showed that prolonged exposure (30 min or 3 hr) of a fish to a jamming stimulus resulted in a sustained JAR. Upon cessation of the stimulus a long-term frequency elevation (LTFE) of the EOD frequency is expressed and over time, gradually relaxes back to baseline (**Figure 5B**).

This phenomenon is a form of sensorimotor adaptation in that the firing frequency of the pacemaker was reset to minimize the effect of the jamming stimulus. LTFE was elicited by sensory stimuli that evoke a JAR and not those that induce bouts of chirping, supporting a role for NMDA, and not AMPA, receptors in the induction of LTFE. *In vitro*, activation of the afferent inputs to the PMn resulted in a fictive JAR followed by LTFE, suggesting that the PMn is the locus of sensorimotor adaptation in this system. (Oestreich and Zakon, 2005).

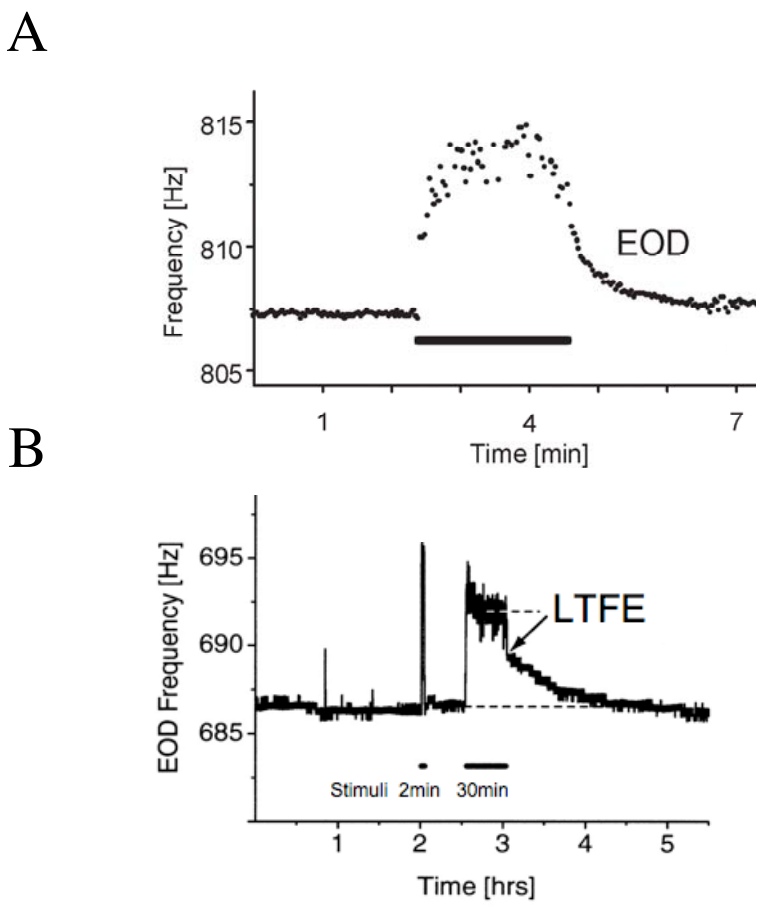


Figure 5: The jamming avoidance response (JAR) and long-term frequency elevation (LTFE) in *A. leptorhynchus*

(A) The *in vivo* experiment demonstrates a fish presented with an artificial sine wave (as indicated by the black bar) 1Hz below its own EOD frequency. Upon presentation of the stimulus, the fish increases its EOD to a new value and maintains the new value for the entire presentation of the stimulus. Upon cessation of the stimulus, the EOD slowly relaxes back to baseline levels. (B) However, a longer stimulus presentation results in LTFE (modified from Bullock et al., 1972; Heiligenberg et al., 1996; Dye, 1987; Oestreich et al., 2005 and (Takizawa et al., 1999).

THE ELECTROMOTOR SYSTEM OF WEAKLY ELECTRIC FISH

During ontogeny, the *Apteronotid* "neurogenic" electric organ develops from nerve cells (**Figure 6**). Although the larvae of these fishes have "myogenic" organs derived from muscle tissue (Kirschbaum, 1983), the adult electric organ is formed by the axons of the electromotor neurons (EMNs) in the spinal cord (Bennett, 1971). In certain

species, the myogenic electric organ is comprised of electrocytes that are innervated by electromotor neurons and the biphasic (and sometimes triphasic) pulse waveform can be modified by the consequences of physiological and morphological innervations of electrocytes (Markham and Stoddard, 2005; Stoddard et al., 2006).

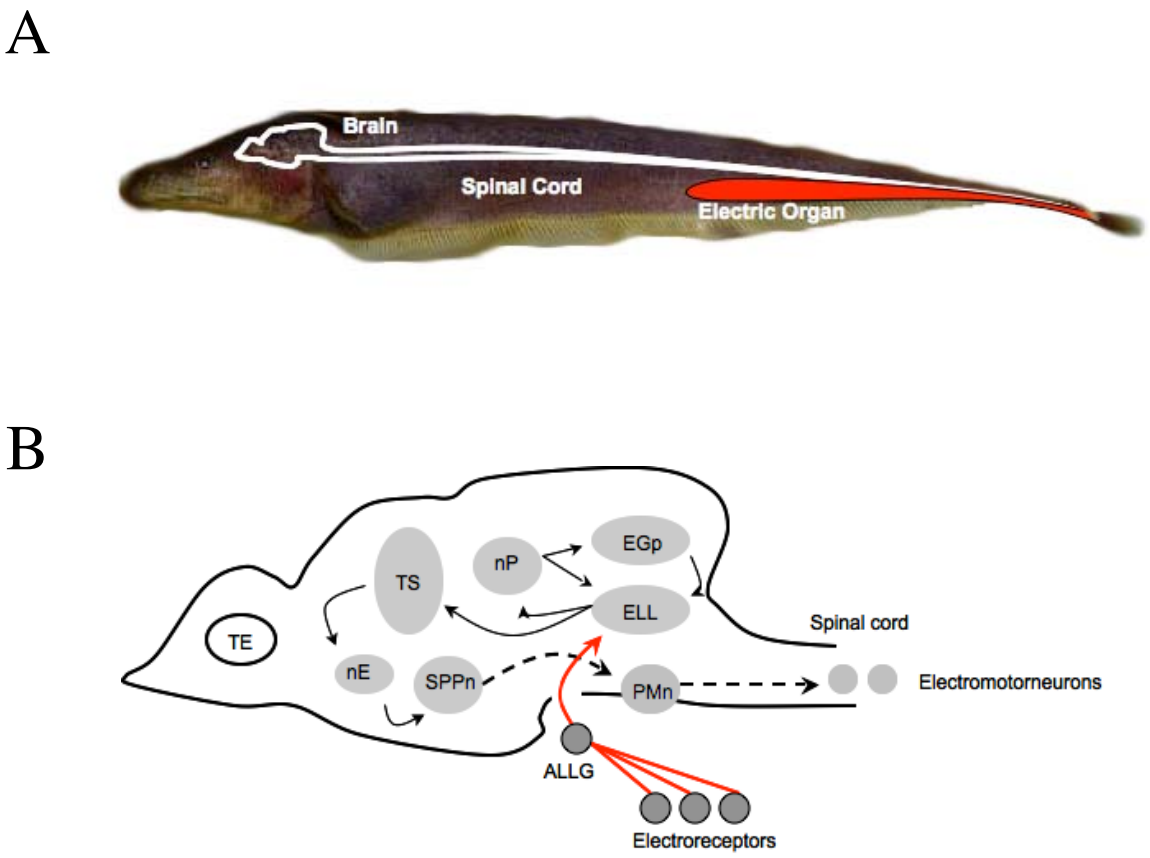


Figure 6: Schematic representation of the electromotor system of *A. leptorhynchus*.

Silhouette of the brown ghost knifefish representing the central nervous system (i.e. brain and spinal cord) and the electric organ (Panel A; colored red). Electrosenory input from electroreceptors (Panel B; red lines) is processed initially in the lobe of the electric lateral line (ELL). Prepacemaker nuclei (sublemniscal prepacemaker nucleus, SPPn) receive input from the nucleus electrosensorius (nE) and send their projections to the medullary pacemaker nucleus (PMn; dashed line). The relay cells within the PMn project axons to the electromotor neurons in the spinal cord (dashed line) and ultimately to electrocytes in the tail (not shown). Abbreviations: Eminentia pars granularis (EGp); nucleus praeminentialis (nP); Torus semicircularis (TS); Telencephalon (TE); Anterior lateral line ganglion (ALLG; Redrawn after (Heiligenberg, 1991a).

The EOD is generated by an autonomously firing nucleus located on the ventral surface of the medulla called the pacemaker nucleus (PMn). The PMn is comprised primarily of two specific cell types: 1) pacemaker neurons that synchronously fire action potentials and set the EOD frequency and 2) relay neurons that are electrically coupled to pacemaker neurons (**Figure 7**). The axons of relay neurons run the length of the spinal cord and electrically synapse on spinal electromotor neurons resulting in the synchronous and unambiguous firing of electric organ (EO).

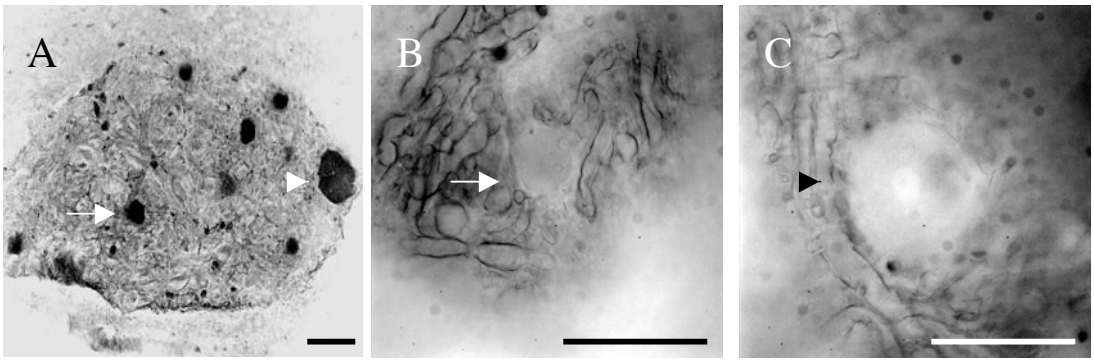


Figure 7: The pacemaker nucleus of *Apteronotus leptorhynchus*.

The *Apteronotus* pacemaker nucleus (PMn) is located on the ventral aspect of the medulla (panel A; transverse section) and is comprised of pacemaker neurons (indicated by arrows) and relay neurons (indicated by arrow heads). Pacemaker neurons (Panel B; indicated by white arrow; transmitted light image) are the intrinsic oscillators of the nucleus and make electrotonic connections with relay neurons (panel C; indicated by black arrowhead; transmitted light image). Scale bars = 100 microns.

CENTRAL MECHANISMS THAT REGULATE THE EOD

As stated previously, the PMn is autonomously firing medullary nucleus that fires *en masse*. The compound action potential generated by the PMn corresponds in a 1:1 fashion with the electric organ discharge but the autonomous activity of the pacemaker alone is insufficient in fully explaining the diverse signals generated by the electric organ. In *A. leptorhynchus,* electrosensory information reaches the electrosensory lateral line lobe, and is then projected to the torus semicircularis in the midbrain (Zupanc and Maler, 1997). From the torus, information is projected to the tectum opticum and the diencephalic nucleus electrosensorius (nE), which acts as an interface between the electrosensory and electromotor system (Keller et al., 1990).

Two specific nuclei within the nE respond to chirp-like stimulus signals and EOD distortions (i.e. beat envelope) caused by the interaction of two neighboring EODs of similar frequency. One subnuclei, the nE↑ sends projections to a pair of diencephalic prepacemaker nuclei (PPn) that mediate gradual rises in the EOD and chirping. These nuclei, known as the PPn-G (for gradual) and PPn-C (for chirping) send projections to the PMn and form chemical synapses with pacemaker neurons (Kawasaki et al., 1988). The other nucleus known as the nE↓ sends projections to different prepacemaker nucleus known as the sublemniscal prepacemaker nucleus (SPPn). The SPPn is a bilateral nucleus located in the mesencephalon (Heiligenberg et al., 1996). These SPPn fiber tracts are glutamatergic and synapse deep within the pacemaker syncytium onto relay and pacemaker neurons. The activation of *N*-methyl-d-aspartate receptors (NMDARs) causes an influx on Ca^{2+} and due to the extensive electrotonic coupling of the PMn; Ca^{2+}

influx leads to a depolarization of the entire nucleus, resulting in the acceleration of PMn firing.

After receiving excitatory input, the SPPn, in turn, projects to the relay cells in the PMn with NMDA receptors mediating the signaling, and glutamate as a neurotransmitter **(Figure 8A)**. Findings suggest that nE↓ tonically inhibits the SPPn by using GABA as a inhibitory neurotransmitter. At the onset of a stimulus signal with a frequency below the fish's own EOD frequency, the inhibition of the SPPn by the nE↓ is removed, presumably because the nE↓ is itself now inhibited by input from the nE↑ (Heiligenberg et al., 1996).

After cessation of the stimulus signal, the EOD frequency falls back to the base level present prior to stimulation due to increasing inhibition of the SPPn either by intrinsic changes in the PMn, increased inhibition by the nE↓, or both. Similar to the SPPn, the PPnC forms synapses on relay cells, with the exception that the glutamatergic input is mediated by AMPA receptors. The PPnG projects onto pacemaker cells and expresses NMDA receptors at the synapses between pacemaker neurons (Heiligenberg et al., 1996). The PPnC controls the fast, transient EOD frequency rises during chirping by mediation over AMPA receptors, which exhibit fast kinetics in other vertebrates, whereas the PPnG like the SPPn is responsible for gradual and slower frequency increases, due to the slower kinetics of the NMDA receptors (Oestreich and Zakon, 2005).

Therefore, the behavioral output of the electromotor system is attributable to a difference in glutamate receptor pharmacokinetics. In *E. virescens,* the nE↑-PPnG circuit controls EOD frequency rises **(Figure 8B)**, whereas the nE↓-SPPn circuit controls EOD frequency falls during JAR (Metzner, 1999). In *E. virescens* the SPPn is tonically active, setting the EOD frequency above a certain base value. Inhibition of the SPPn by the nE↓ causes a deceleration in PMn firing.

E. virescens is able to respond to a jamming signal with a frequency below its

own EOD frequency (negative difference frequency; -df) with a frequency increase during the JAR (Metzner, 1999) and a jamming signal with a positive difference frequency (+df) decreases in EOD frequency during the JAR. The SPPn in *A. leptorhynchus* is not tonically active and therefore *A. leptorhynchus* is not able to decrease its EOD frequency below the resting level (Oestreich and Zakon, 2005). The nE↓-SPPn pathway only reduces the EOD frequency when the frequency is already elevated during a JAR, due to a stimulus with -df. The nE↑-PPnG in *A. leptorhynchus* leads to an gradual increase in EOD frequency during stimulus presentation with a +df, but does not contribute to the JAR during stimulus presentation with a negative df, because the nE↑ receives some suppression during activity of the nE↓, which is preferentially active during the presence of signals with a negative df (Heiligenberg et al., 1996).

The SPPn might also contribute to the production of yodels or EOD interruptions, as they can be seen during intensive courtship and spawning, because a direct, strong stimulation of the SPPn can mimic these behavioral signals (Heiligenberg et al., 1996). Reasons for this might be that *E. virescens* is active in a lower frequency range (250-600 Hz) than *A. leptorhynchus* (600-1000 Hz) and that adaptations, allowing synchrony at high frequencies exclude the existence of a tonically active input (Heiligenberg et al., 1996).

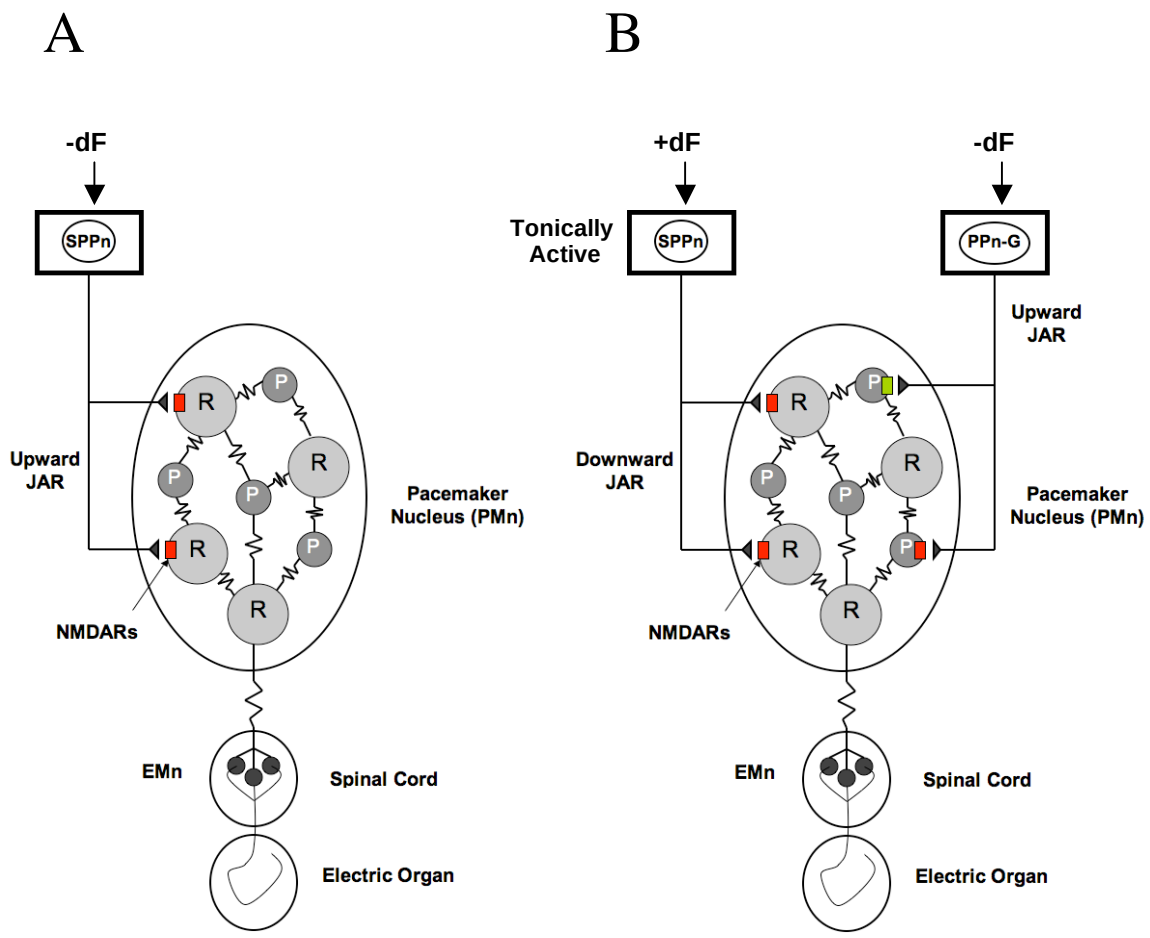


Figure 8: Schematic diagrams of the control circuitry of *A. leptorhynchus* and *E. virescens*.

In *Apteronotus* (A), an upward JAR is activated by the presence of a stimulus signal with negative difference frequency (-dF) through the activation of the SPPn and subsequent activation of NMDA receptors (red boxes) expressed on relay neurons. The acceleration in PMn firing rate is conveyed down electromotor neurons in the spinal cord to the electric organ. In *Eigenmannia* (B), a -dF activates the PPn-G, activating AMPARs (green boxes) and NMDARs (red boxes) expressed on pacemaker neurons resulting in PMn frequency acceleration. In contrast, a +dF signal inhibits the SPPn, which otherwise provides tonic input to the PMn via NMDARs expressed on relay neurons. This will cause a downward JAR. Stimulus frequency below or above EOD frequency (dF, +dF), SPPn sublemniscal prepacemaker nucleus (SPPn), thalamic prepacemaker nucleus

portion G (PPn-G), PMn pacemaker nucleus (PMn), pacemaker cell (P), relay cell (R). (Modified from (Heiligenberg et al., 1996; Oestreich and Zakon, 2005).

WEAKLY ELECTRIC FISH AS A MODEL SYSTEM

Electric fish have proven to be the quintessential model system for studying the central mechanisms for generating motor output. Many vertebrate behaviors involve a myriad of complex interacting elements, making it difficult to study the neural correlates of behavior (Bullock, 1982). Electric fish have evolved electrical fields that allow them to communicate with one another and to detect objects that have different impedance than the surrounding water. The electromotor output of mormyriforms and gymnotiforms is characterized by electric organ discharges (EODs) that are species-specific and often sexually dimorphic.

The EOD is highly stereotyped (characterized by just two parameters: the EOD waveform and its temporal pattern (Bullock, 1973; Heiligenberg, 1991b) and controlled by a few discrete nuclei in the brainstem and spinal cord all the way to the electric organ. The organization makes it an exquisite system for investigating how long-term changes in sensory input modulate behavior, neural circuitry, hormone responses, and ion channels (Dulka et al., 1995; Dye, 1988; Meyer et al., 1987a; Schaefer and Zakon, 1996).

The EOD represents a repertoire of the animal's behavior that is readily quantifiable (Metzner and Heiligenberg, 1991; Oestreich and Zakon, 2002). The electrical signals are the 'currency' of the nervous system and, therefore, species-specific differences in sex (and individual differences) are manifestations in differential neural wiring and reflect a small number of brainstem and thalamic nuclei on a single electrotonically coupled system where the temporal properties of the EOD are integrated (Hopkins, 1986). Coupled with *in vivo* and *in vitro* electrophysiological techniques, the

behavioral plasticity observed in these animals can be correlated with the cellular and molecular mechanisms and, more precisely, how these mechanisms contribute to adaptive changes in neural excitability.

ACTIVITY DEPENDENT CHANGE IN PACEMAKER FIRING RATE

Activity dependent change in neural plasticity is a broad term that includes learning, but it also includes other phenomena such as the adaptation of neural circuits to altered forms of sensory input (Zhang and Linden, 2003). The changes can be morphological changes in neural architecture (e.g. dendritic and spine growth) as well as intrinsic changes (e.g. changes in threshold and/or changes in current flow through voltage-gated channels). Plasticity of neural circuits do not always result from activity dependent changes but can occur from trauma to a particular sensory modality (Darlington et al., 2002).

One example is the compensation that occurs within vestibular systems when one vestibular labyrinth or vestibular nerve is damaged or is rendered non-functional due to trauma. The damage results in the inappropriate activation of vestibulo-ocular and vestibulo-spinal reflexes (Cameron and Dutia, 1999; Darlington et al., 2002; Dutia, 2000) and occurs largely as the result of the asymmetry in the basal firing rate of neurons in the vestibular nucleus. However, these symptoms are reduced over time, and this is correlated with an increase in the basal firing rate of neurons in the vestibular nucleus on the lesioned side. This increase in excitability is due to an increase in the basal firing rate, concomitant with an increase in the resting potential and in the amplitude of a fast afterhyperpolarization (Straka et al., 2005).

Activity dependent change in neural excitability has been demonstrated in the PMn slice preparation of *A. leptorhynchus* (Dye, 1991). Initially, brief electrical stimulation of the prepacemaker fibers elicited short-latency accelerations in pacemaker frequency that represented the in vitro expression of specific behaviors such as chirping (Dye, 1991). However, tetanic stimulation of the afferent fibers increases PMn spike frequency (representing a fictive jamming avoidance) and the new firing rate remains elevated above baseline for minutes. This phenomenon has been termed Long-Term Frequency Elevation (LTFE) and is a form of sensorimotor adaptation in that the pacemaker firing frequency was reset in response to a long jamming stimulus. These long lasting changes are the basis for sensorimotor adaptations whereby persistent changes in motor output result from persistent changes in sensory input. The usual stimulus paradigm used to study the JAR in vivo was to give the fish a stimulus of up to 2 min, after which its EOD frequency returns to its baseline value (Bullock et al., 1972a; Dye, 1987; Dulka and Maler, 1994; Heiligenberg et al., 1996; Takizawa et al., 1999).

Recent experiments have demonstrated that prolonged exposure of a fish to a jamming stimulus results in a sustained JAR. Upon cessation of the stimulus, a long-term frequency elevation of the EOD frequency lasts for hours and gradually relaxes back to baseline (Oestreich and Zakon, 2002). More importantly, LTFE is elicited by sensory stimuli that evoke a JAR and not those that induce bouts of chirping. This corroborates the findings that NMDA, and not AMPA receptors are responsible for the induction of LTFE. *In vitro*, activation of the afferent inputs to the PMn resulted in a fictive JAR followed by LTFE, suggesting that the PMn is the locus of sensorimotor adaptation in this system (Oestreich and Zakon, 2002).

Chapter 3: Synaptically Induced Long-Term Frequency Elevation in *A. leptorhynchus* is Regulated by Calcium-Dependant Phosphorylation

ABSTRACT

The purpose of this study was to examine whether calcium-mediated phosphorylation plays a role in the induction and/or maintenance of LTFE. Using an *in vitro* brain slice preparation from *A. leptorhynchus*, I tested the hypothesis that two major effectors of calcium-dependent phosphorylation, PKC and calcineurin (PP2B), play important roles in maintaining LTFE. I demonstrate in the following sets of experiments that the phosphorylation/dephosphorylation events mediated by these two enzymes influence the persistent activity of the pacemaker nucleus in response to synaptic input and, more importantly, do not require CaMKII. I also show that the inhibition of two isoforms of PKC, PKCα/β and PKCζ, attenuate the maintenance of LTFE while the inhibition of calcineurin enhances the maintenance of LTFE. In addition, I demonstrate that conventional and atypical PKCs work cooperatively in regulating spontaneous activity of PMn neurons and contribute to our understanding behind the mechanisms that underlie the species-specific sex differences in the EOD.

INTRODUCTION

NMDA receptor-initiated changes in synaptic strength as a mechanism for temporarily storing and manipulating information have been extensively studied (Kapur et al., 1997; Seamans et al., 2003; Wang, 1999; Yoshida et al., 2008) with less attention

devoted to the role of NMDA receptor-initiated long-term changes in intrinsic excitability. A key question is whether the cellular pathways activated by NMDA receptors to modify intrinsic excitability (plasticity) are the same as or different from those involved in maintaining a neuron's basal state of excitability (homeostasis).

The weakly electric fish, *Apteronotus leptorhynchus*, generates electric fields around its body through a specialized electric organ and senses these electric organ discharges (EODs) for electrolocation and electrocommunication (Hopkins et al., 1997). The EOD represents one of the most stable biological oscillators known with submicrosecond precision (Bullock, 1969; Moortgat et al., 2000; Zakon et al., 2002). Within this species, EOD frequency is individual- and, gender-specific (Hopkins, 1988). Each fish retains its particular EOD frequency for days when placed in social isolation (J. Oestreich, unpubl.). The EOD frequency is established by an autonomously firing medullary pacemaker nucleus (PMn) that is comprised primarily of two specific cell types: 1) pacemaker neurons, which are the internal oscillators and set the PMn firing rate and 2) relay neurons, which send their axons out of the PMn to innervate spinal motorneurons. **(Figure 9)**. Pacemaker and relay cells are coupled by gap junctions and entrain each other. Each action potential from the pacemaker elicits an action potential from the electric organ so EOD frequency is determined in a 1:1 fashion by PMn firing frequency.

Despite this extreme stability, the EOD frequency shows a form of graded nonassociative memory that underlies sensorimotor adaptation in the electromotor system of a weakly electric fish (Oestreich et al., 2006). If two fish with almost identical frequencies meet, their signal interaction leads to ’beating’ of their EOD waveforms and this and jams their electrosensory systems. Both fish detect this interference pattern and usually the fish with the slightly higher EOD frequency prevents jamming by transiently

raising its discharge frequency, a behavior known as the jamming avoidance response (JAR). The JAR is generated by the recruitment of normally quiescent, descending inputs from a higher-order nucleus (the sublemniscal prepacemaker nucleus; SPPn) that activates *N*-methyl-d-aspartate receptors (NMDARs) expressed in pacemaker and relay neurons (Harvey-Girard et al., 2007; Metzner, 1993). Exposure to a jamming stimulus for many minutes results in a prolonged JAR and, upon cessation of the jamming stimulus, a long-term frequency elevation (LTFE) of the fish's EOD persists for tens of minutes or hours (Oestreich and Zakon, 2005).

In many forms of neuronal plasticity (Belousov et al., 2002; Buonomano and Byrne, 1990; Connor et al., 1999; Eccles, 1983; Toth et al., 2000; Xu et al., 2005), the activation of NMDARs followed by an the influx of Ca^{2+} is the critical link between brief synaptic input and long-term responses (Abel et al., 1998; Egorov et al., 2002; Gray et al., 1996; Otmakhov et al., 2004). The Ca^{2+} signals generated by influx and mobilization from internal stores are converted into physiological responses through several Ca^{2+}-sensing molecules. Ca^{2+} can activate kinases such as protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and the calmodulin-dependent protein phosphatase calcineurin (Qu et al., 1994).

These effectors regulate neural signaling by acting as positive and negative regulators of cellular activity through the phosphorylation or dephosphorylation of molecular targets (Rycroft and Gibb, 2004; Schacher et al., 1990; Xu et al., 2005). In LTFE, NMDAR-mediated Ca^{2+} influx is directly responsible for changes in the excitability of PMn neurons. (Oestreich et al., 2006). However, the downstream signaling pathways and molecular targets of Ca^{2+} have not been identified.

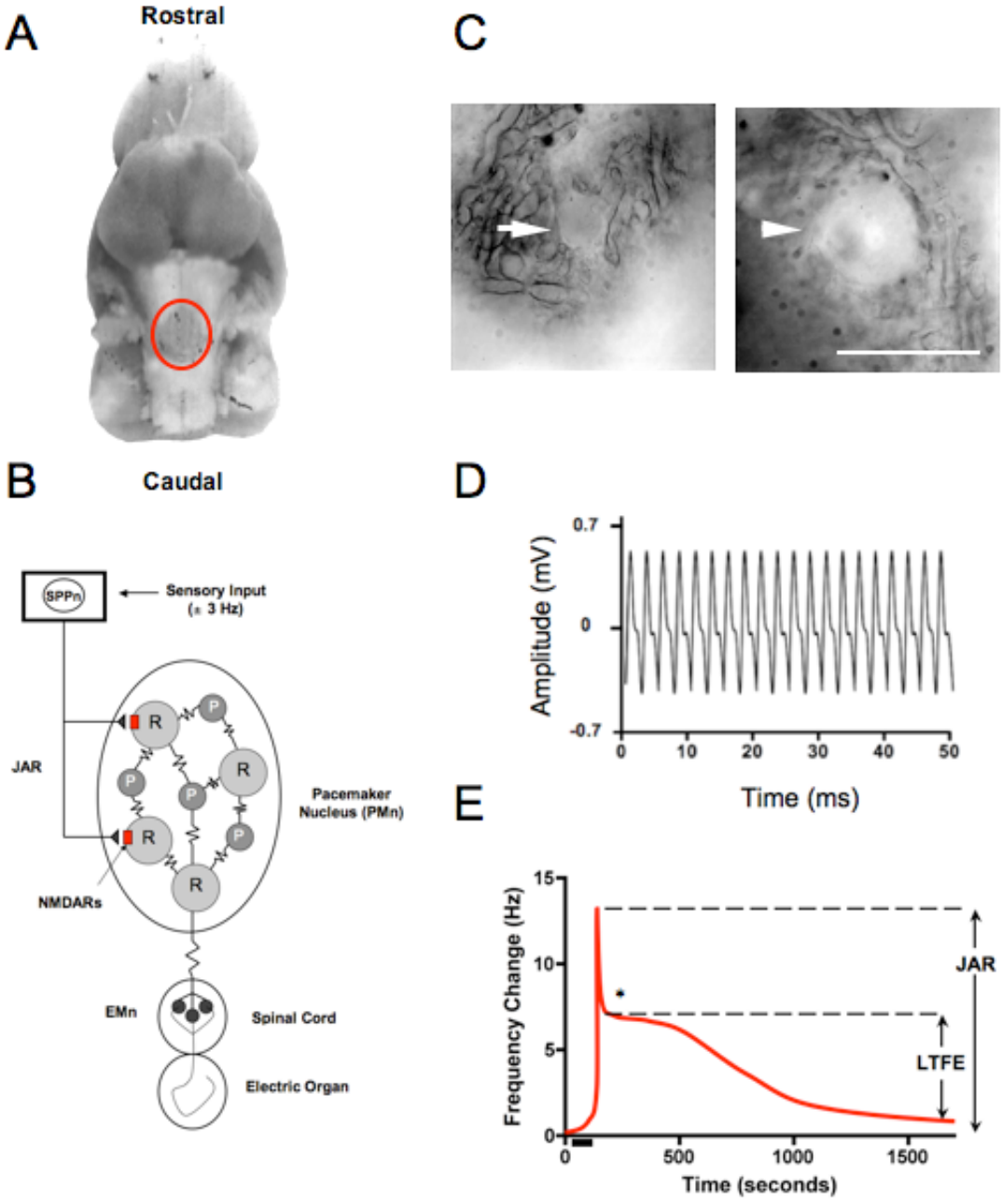

A
Rostral
B
Caudal
SPPn
Sensory Input
(± 3 Hz)
JAR
R
P
Pacemaker
Nucleus (PMn)
NMDARs
EMn
Spinal Cord
Electric Organ
C
D
Amplitude (mV)
0.7
0
-0.7
0
10
20
30
40
50
Time (ms)
E
Frequency Change (Hz)
15
10
5
0
JAR
LTFE
0
500
1000
1500
Time (seconds)

Figure 9: Organization of the electromotor system of A. leptorhynchus and the principle of long-term frequency elevation (LTFE).

(A) The pacemaker nucleus (PMn) can be visualized on the ventral surface of the caudal medulla (circled in red). (B) Schematic representation of the pacemaker nucleus. Pacemaker and relay neurons (labeled P and R respectively) are electrotonically coupled to one another and autodepolarize *en masse*. Glutamatergic inputs from sublemniscal prepacemaker nuclei (SPPn) activate NMDA (N-methyl-D-aspartate) receptors expressed on relay neurons and mediate the upward jamming avoidance response (JAR). Relay neurons are electrotonically coupled to electromotor neurons (EMN) located within the spinal cord and convey information to the electric organ in the tail. (C) Transmitted light images of pacemaker (arrowhead) and relay (arrow) neurons of the PMn. Scale bar = 100 microns. (D) The electric organ discharge (EOD) of *A. leptorhynchus*. (E) LTFE recorded from the isolated pacemaker nucleus. After recording a stable baseline frequency, stimulation (solid black line) of the SPPn afferent fibers results in a rapid acceleration in PMn frequency (fictive JAR). Stimulus cessation leads to rapid declination in frequency to a new stable value (asterisk indicating LTFE induction) and subsequent maintenance of LTFE.

In the present study, I will use an *in vitro* brain slice preparation from *A. leptorhynchus* to test the hypotheses that Ca^{2+}/calmodulin dependant protein kinase II (CaMKII), protein kinase C (PKC), and calcineurin (protein phosphatases 2B or PP2B) play a role in 1) neural homeostasis - manifest as constancy of the PMn frequency and 2) neural plasticity- manifest as the induction and/or maintenance of LTFE. Combined with calcium imaging, we will test whether LTFE builds upon the same mechanisms that are used to regulate pacemaker spontaneous activity.

MATERIALS AND METHODS

Animals

In accordance with the University of Texas at Austin's IACAUC animal protocols, wild-caught individuals of the weakly electric fish *Apteronotus leptorhynchus* were obtained through Segrest Farms (Gibsonton, FL) and housed in community tanks in climate-controlled rooms (stable between 26ºC and 28ºC) and under a 12 hr dark/ light cycle. Fish were fed with frozen brine shrimp every 2 days. Individuals were allowed to acclimatize for 1 month before being used in experiments with water conductivity held at 800 uS/cm.

PMn Slice Preparation

The *Apteronotus* PMn slice preparation **(Figure 10)** has been previously described (Dye, 1987; Oestreich and Zakon, 2002). Briefly, fish were anesthetized in 0.1% 2-phenoxyethanol (Sigma, St. Louis, MO), positioned on ice, and brains were irrigated with ice-cold cold artificial cerebral spinal fluid (ACSF; see below) and removed immediately. Whole brains were transferred to Sylgard-coated Petri dishes containing oxygenated (95% O2–5% CO2) ice-cold artificial cerebral spinal fluid (ACSF: 124 mM NaCl, 2 mM KCl, 1.25 mM KH2PO4, 1.1 mM MgSO4, 1.1 mM CaCl2, 18 mM NaHCO3, and 10 mM glucose). Subsequently, brains were pinned down, ventral side up, and meninges were removed from the brainstem. The ventral surface of the brainstem, spanning from a point in close proximity of the caudal aspect of the pacemaker nucleus to the pituitary fossa, was dissected from the rest of the brain (thickness ~700 μm). Care

was taken to incorporate the afferent fibers from the prepacemaker nuclei that run close to the ventral surface of the medulla (Dye, 1988; Maler et al., 1991; Zupanc and Horschke, 1997).

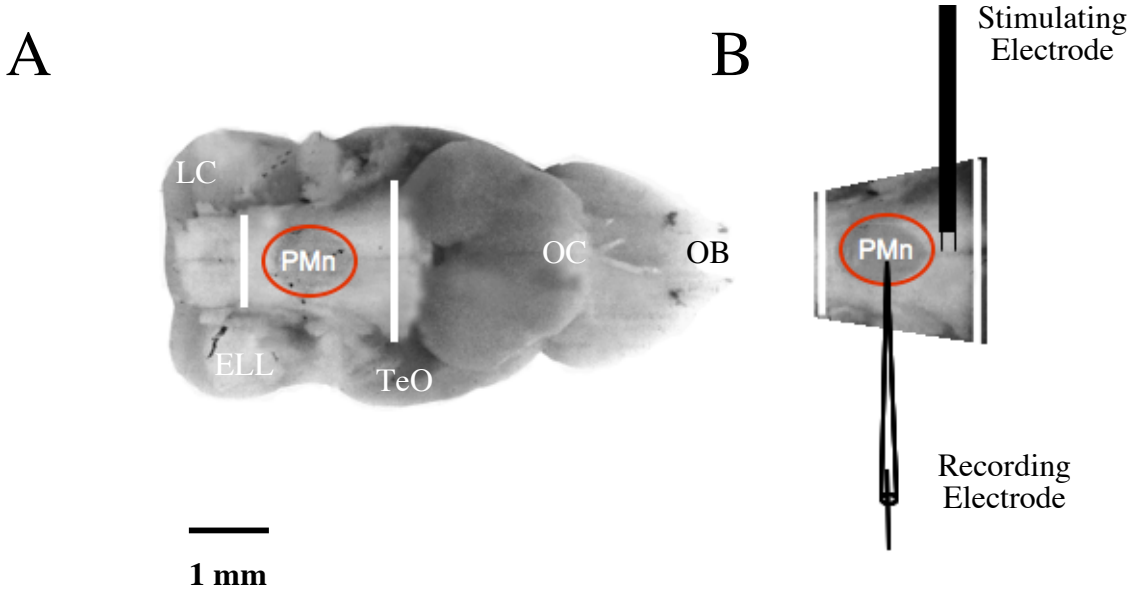


Figure 10: Ventral view of the brain of *A. leptorhynchus*.

The pacemaker nucleus (PMn) is located on the ventral surface of the caudal medulla (Panel A; red circle). The prepacemaker nuclei are located more dorso-rostral in the diencephalon and mesencephalon and are not included in the slice preparation. The PMn slice preparation is prepared by excising the ventral brain stem from the rest of the brain is indicated by the lines and carefully placing a bipolar stimulating electrode on the glutamatergic tract from the SPPn and glass microelectrode filled with 1M NaCl to record the PMn field potential (Panel B). Abbreviations: ELL - electrosensory lateral line lobe, LC - caudal lobe of the cerebellum, OB - olfactory bulb, OC - optic chiasm, PMn - pacemaker nucleus, TeO - optic tectum. Scale bar = 1 mm. Modified from: (Maler et al., 1991).

PMn Recordings

PMn slices were transferred to a Plexiglas recording chamber (designed by R. Turner and L. Maler (The University of Calgary, Calgary, Alberta, Canada and the University of Ottawa, Ottawa, Ontario, Canada) and oxygenated ACSF was bath perfused (flow rate = 12ml/min) continuously by means of a peristaltic pump (Dynamax RP-1; Rainin, Emeryville, CA). Bath temperature was tightly regulated (TH-10Km thermisotor probe and TC2 temperature controller; Cell Micro-Controls, Norofolk, Virginia, USA) and maintained at 25°C **(Figure 11)**.

Glutamatergic fiber tracts to PMn were activated with bipolar matrix microelectrodes (FHC, Bowdoin, ME, USA) under the following stimulus parameters: Train duration: 1 second, pulse width: 400 μs, rate: 500 Hz, and stimulus amplitude of 40 uA (Grass Stimulator, model S88; Grass Technologies, West Warwick, RI, USA). In order to avoid saturation by overstimulation and to allow for recovery of LTFE in enough time to carry out multiple trials, current injection of 40 uA was used to elicit LTFE.

An extracellular recording electrode (filled with 1M NaCl) was positioned (Sutter MP-285 micromanipulator, Novato, CA) directly above the pacemaker nucleus until the pacemaker field potential was detected with an oscilloscope. PMn field potential was amplified using an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA) and an A-M Systems model 1700 differential AC amplifier (Carlsborg, WA). Analog to digital conversion was accomplished using a Digidata 1320 series digitizer (Molecular Devices, Sunnyvale, CA) and recorded with pClamp software (Molecular Devices, version 8).

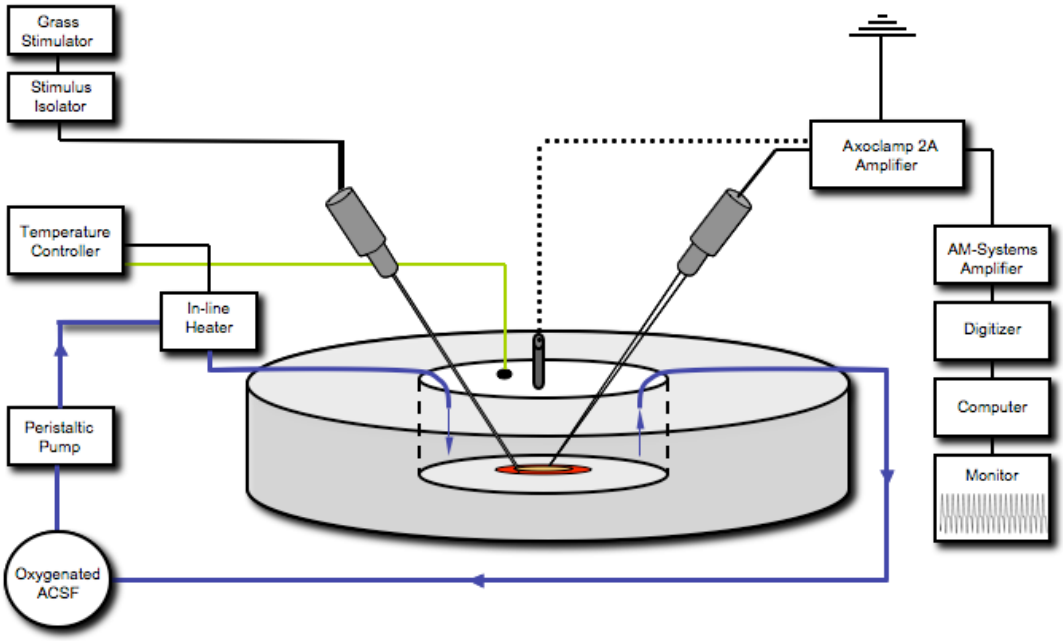


Figure 11: Schematic representation of the *in vitro* recording chamber.

Pacemaker slices (indicated by orange oval) were placed in an illuminated Plexiglas chamber and artificial cerebral spinal fluid (ACSF) was perfused under constant oxygenation (95%O_2/5%CO_2) via peristaltic pump (12ml/min; indicated by blue line). Temperature was closely monitored though a thermistor temperate probe (indicated by green line; see methods) and adjusted precisely through an in-line heater (25^{0}C). Electrical stimulation of the SPPn prepacemaker fibers to the PMn was performed using a Grass Stimulator and PMn field potential was recorded with a glass mircroelectrode amplified with an Axoclamp 2A amplifier (reference indicated by dotted line).

Stimulation of the afferent fiber tract to PMn produces a fictive JAR that is indicative of NMDAR activation and calcium influx into relay neurons. Therefore, we define the induction of LTFE arbitrarily at 40 seconds after stimulation. This time point is an indication that the mechanisms that regulate the JAR have occurred completely intact. LTFE maintenance is then defined as the duration of the frequency elevation after LTFE induction. For experiments that required stimulus modifications to match LTFE

induction values, stimulus amplitudes were adjusted between 40 uA and 60 uA with all other stimulus parameters held constant.

Pharmacology

Calmidazolium chloride, cyclosporin A and KN-62 were obtained from Tocris Biosccience (Ellisvile, MO) and the cell-permeable PKC inhibitors (Myr-PKCα/β; myristolated psuedosubstrate sequence from PKCα and PKCβ, N-Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln) and cell permeable PKCζ inhibitor (Myr-PKCζ; myristolated pseudosubstrate sequence from PKCζ, N-Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu) were purchased from BioMol International (Plymouth Meeting, PA). These compounds were diluted in oxygenated ACSF and then applied to PMn slice preparations. FK506 was purchased from A.G. Scientific Inc. (San Diego, CA). Cyclosporin A, FK506, and okadaic acid (EMD Biosciences, Inc. San Diego, CA) were dissolved in DMSO and bath applied to PMn preparations with a final DMSO concentration of 0.1%. Controls experiments were performed with 0.1% DMSO.

Simultaneous PMn recordings and Calcium Imaging

Pacemaker recordings were carried out (as describe above) concomitant with pacemaker and relay neurons iontophoretically microinjected with Fura-Dextran (dissolved in 200 mM KCl) and cytosolic calcium transients were captured on an Olympus (Olympus - BX51) microscope with Olympus Optical (Tokyo, Japan) water-immersion objective (40X, 0.9NA). The microscope was fitted with an Andor iXon DV887 EMCCD camera and controlled by iQ Imaging Software (Andor Technologies,

Belfast, Ireland). Images were acquired every 2 seconds through a 510/84 nm bandpass filter as a mercury arc lamp illuminated slice preparations alternately through 340/26 nm and 387/11 nm bandpass filters (Semrock, Rochester, NY). Ratiometric values indicating relative cytosolic calcium levels were calculated by dividing the emission intensity at 510/84 nm when excited at 340/26 nm by the emission intensity at 510/84 nm when excited at 387/11 nm.

Statistical Analysis

All statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software Inc., San Diego CA). Means and standard error of the mean were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Groups were prepared using unpaired Student's t test and repeated measures analysis of variance with significance determined by $p < 0.05$. For linear regression, goodness-of-fit (r^2 and F values) and significance were determined using Graphpad's built-in linear regression functions. Multiple regression analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL).

RESULTS

CaMKII Does Not Contribute to PMN Firing Rate or LTFE

To examine whether CaMKII, is required for the induction and/or maintenance of LTFE, we pretreated PMn slices with the specific CaMKII inhibitor KN-62 (10 uM). KN-62 did not affect PMn basal firing rate (**Figure 12A**, n.s.), maintenance or induction of LTFE (**Figure 12B and B'** respectively, $p > 0.05$).

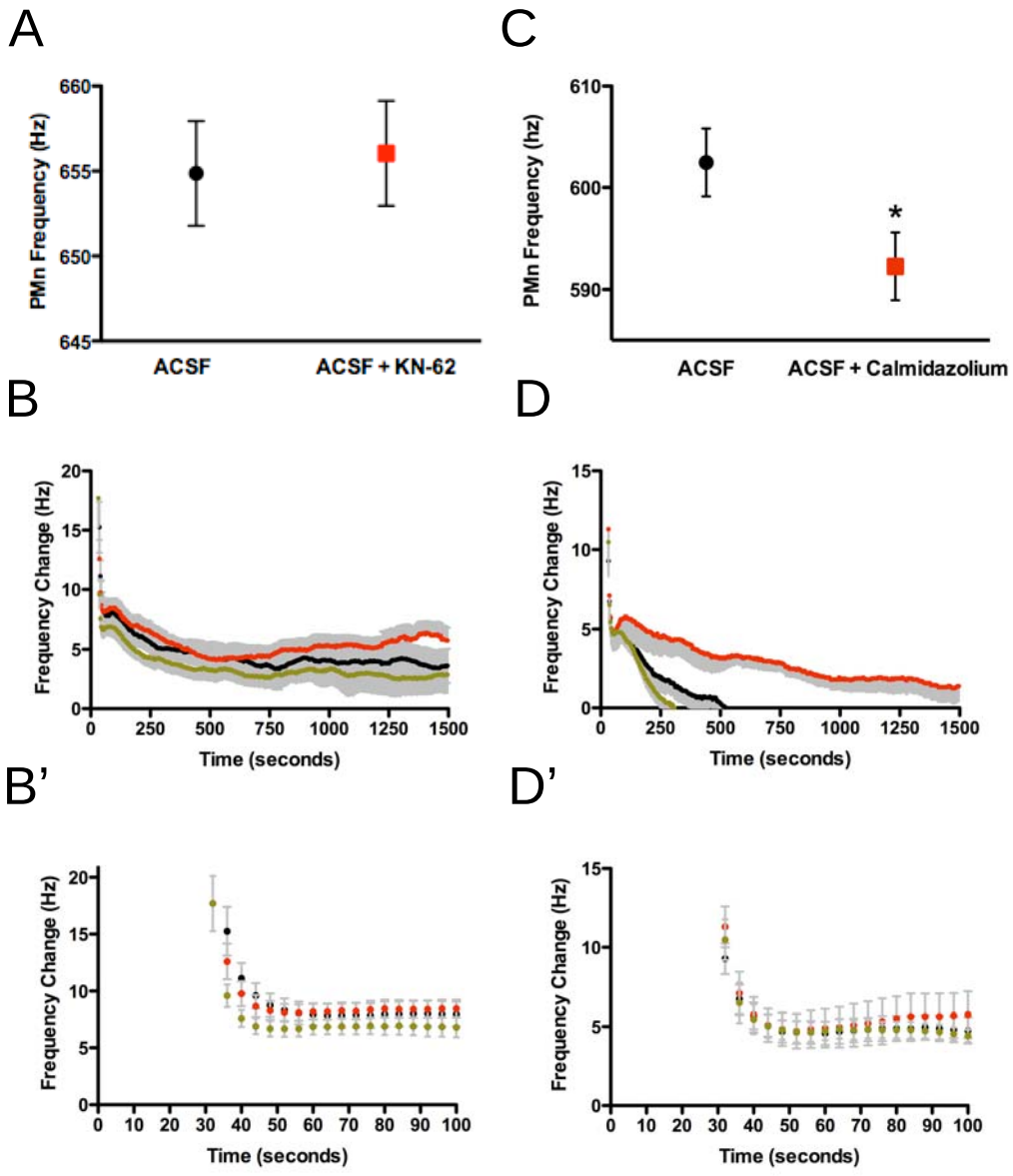


Figure 12: Ca^{2+}/calmodulin-dependant kinase II (CaMKII) is not necessary for LTFE.

(A) Application of a specific CaMKII inhibitor, KN-62 had no effect on PMn basal firing rate (● ACSF alone, ■ KN-62, 10 uM; n.s.), LTFE maintenance or LTFE induction (B and B' respectively; ● initial stimulation, ● KN-62, ● wash stimulation; n.s.). (C) Calmidazolium chloride, a specific calmodulin antagonist, decreased PMn basal firing (● ACSF alone, ■ calmidazolium chloride, 30 uM; $p < 0.05$) and enhanced LTFE maintenance (D; $p < 0.001$) with no change in induction (D'; ● initial stimulation, ● calmidazolium, ● wash stimulation; n.s.).

Since CaMKII is activated by CaM, blocking CaM should also eliminate LTFE if CaMKII initiates LTFE. To test this we pretreated PMn slices with a specific calmodulin antagonist, calmidazolium chloride (30uM). After superfusion of calmidazolium, PMn basal firing rate decreased by 10.2 Hz ± 3.3 Hz, representing an average reduction of 1.7% (**Figure 12C**, $p < 0.05$). Paradoxically, inhibition of CaM did not eliminate, but rather enhanced LTFE maintenance (**Figure 12D**, $p < 0.001$) with no change in LTFE induction (Fig. 12D', n.s.).

LTFE Requires Conventional Isoforms of Protein Kinase C (PKC)

We tested whether conventional PKCs are involved in LTFE by pretreating slices with a highly specific myristolated protein kinase C inhibitor (Myr-PKCα/β; psuedosubstrate sequence from PKCα and PKCβ; 10nM). Because we are unable to differentiate between the enzymatic activity of PKCα and PKCβ in this study, we use the term PKCα/β to refer to the possibility that either or both enzymes may be involved. PMn basal firing rate decreased by an average of 48.2 Hz or 8.8% ± 1.5% (**Figure 13A**, $p < 0.001$). Inhibition of conventional PKC isoforms resulted in a decrease in the maintenance of LTFE (**Figure 13B**; $p < 0.001$) and LTFE induction (**Figure 13B'**; 9.5 Hz to 5.2 Hz ± 1.6 Hz).

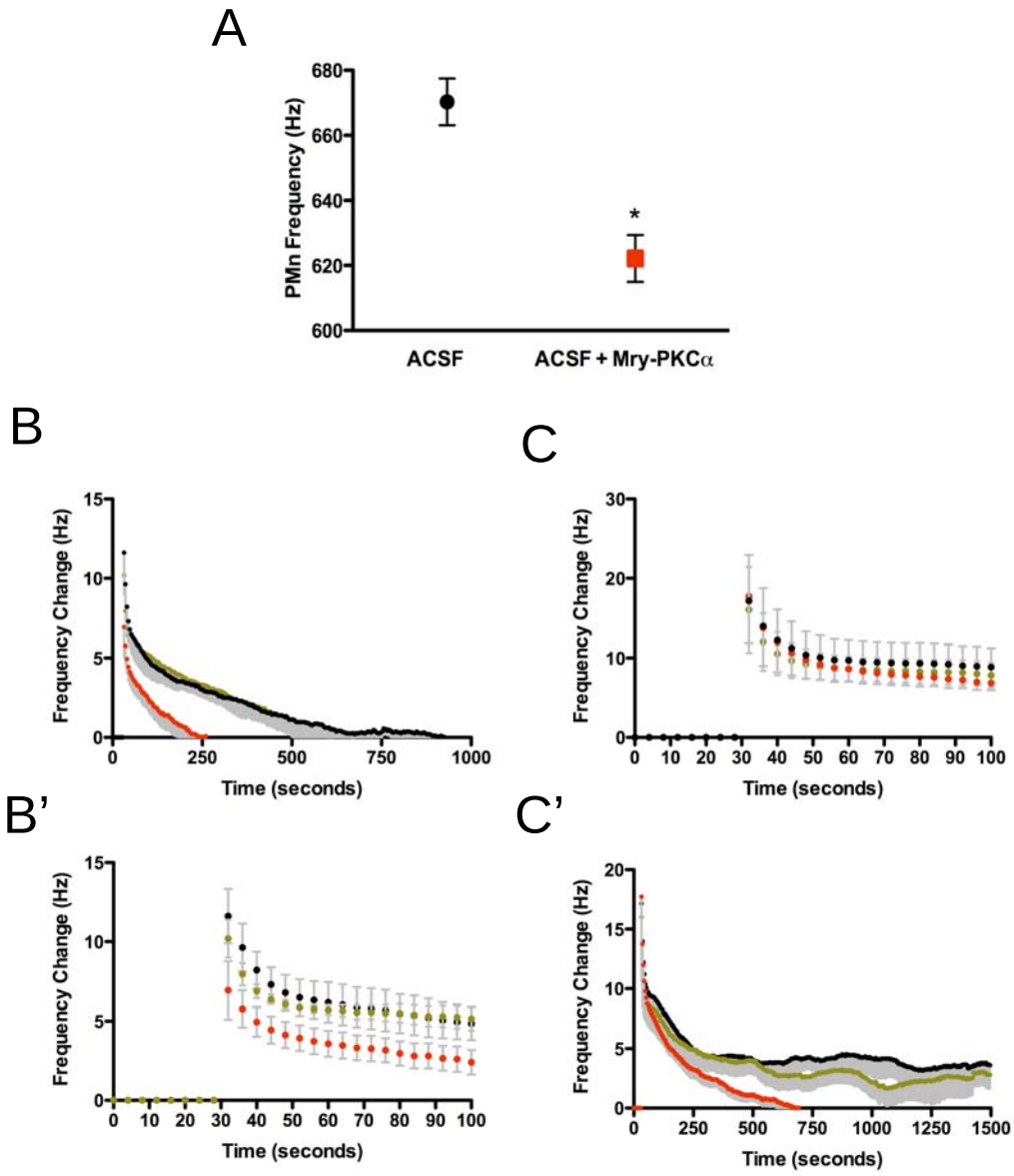


Figure 13: Inhibition of conventional PKCs lowers basal firing rate and attenuates LTFE.

(A) Application of a specific PKC antagonist (myristolated PKC oligopeptide generated against the catalytic domains of PKCα and PKCβ) significantly attenuates PMn basal firing (● ACSF alone, ■ Myr-PKCα/β, 10 nM $p < 0.001$), LTFE maintenance and induction (B and B' respectively; ● initial stimulation, ● Myr-PKCα/β, ● wash stimulation; $p < 0.001$). (C) Upon increasing stimulus intensity of glutamatergic afferents to the pacemaker (see methods), no differences in LTFE induction were observed ($p > 0.05$). However, LTFE maintenance was still attenuated ($p < 0.001$).

Because bath-applied agents might act presynaptically and/or postsynaptically, the attenuation in maintenance of LTFE could be explained by reduced induction of LTFE through decreased presynaptic release or postsynaptic response to glutamate. Therefore, we increased the stimulus intensity to the glutamatergic afferents that synapse within the PMn (see methods) to match LTFE induction values (**Figure 13C**, n.s.). The conventional PKC antagonist still resulted in a significant attenuation of LTFE (**Figure 13C'**, $p < 0.001$). These results support our hypothesis that regulation of LTFE maintenance is mediated by PKC within pacemaker and relay neurons.

The previous experiment showed that reduction of LTFE by PKC inhibitors is not due to a decrease in transmitter release or amount of postsynaptic depolarization by the transmitter. However, since Ca^{2+} is not a major charge carrier through the NMDA receptor (Tsien and Tsien, 1990), if PKC antagonists decrease the Ca^{2+} permeability of the NMDA receptor we would be unable to detect it by monitoring firing rate alone. Furthermore, PKC blockers might also influence the regulation of intracellular stores of Ca^{2+}. To determine whether the inhibition of PKC altered Ca^{2+} influx through NMDARs and/or efflux from internal stores, relay and pacemaker neurons were loaded with fura-dextran (**Figure 14A**) and relative cytosolic Ca^{2+}transients were imaged while simultaneously recording PMn activity.

Superfusion of the conventional PKC inhibitor reduced the magnitude of Ca^{2+} after stimulation by 46% (**Figure 14B**; $p < 0.001$) and the total Ca^{2+} (measured by the integral after induction) by 66% (**Figure 14C**, $p < 0.001$). To test whether this was solely due to the reduction in synaptic transmission caused by PKC inhibition, we increased the stimulation intensity as above.

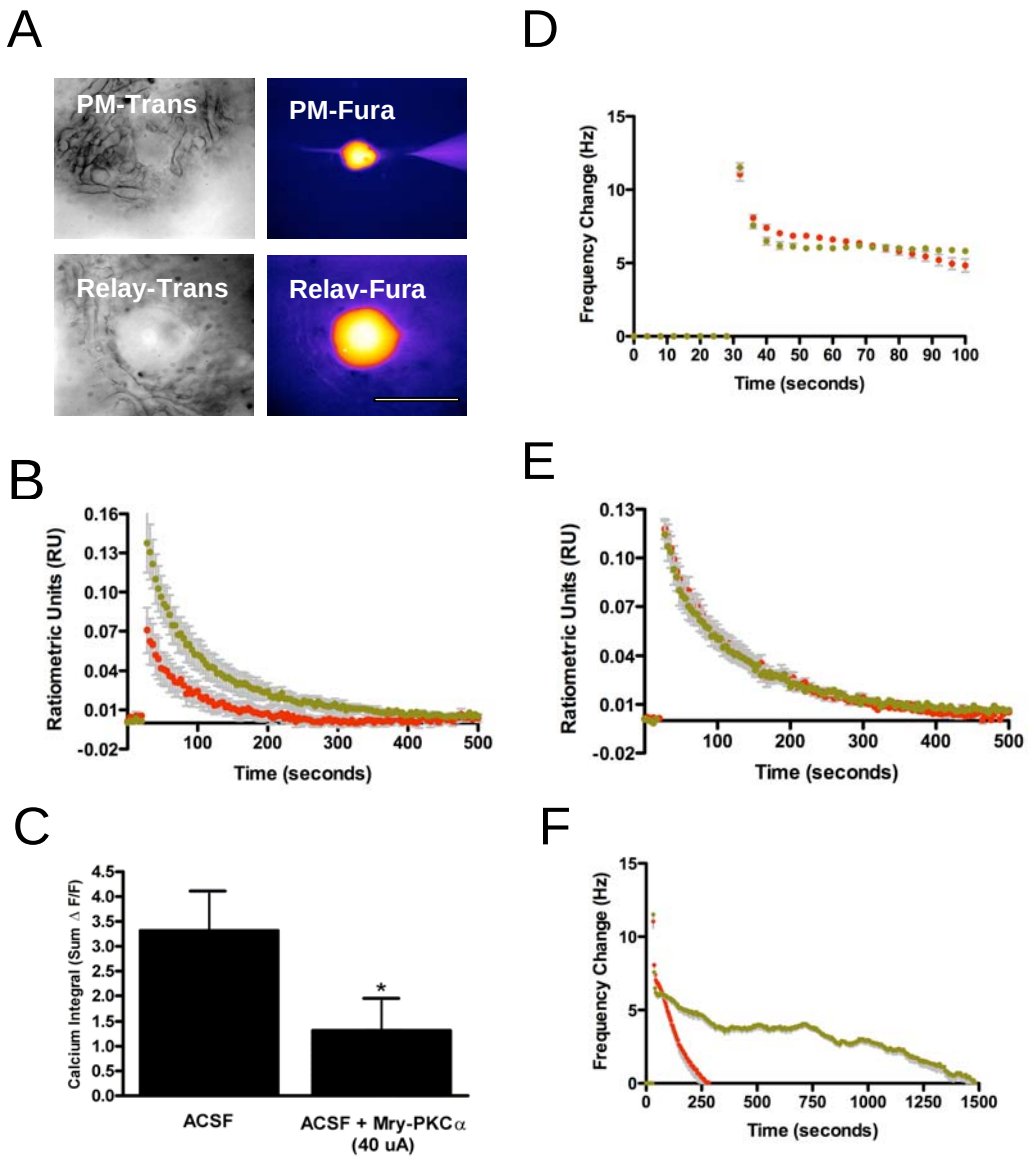


Figure 14: The effects of PKC inhibition on calcium signaling in the PMn.

(A) Pacemaker and relay neurons (transmitted light images on left) were loaded with fura-dextran to measure the effects of PKC inhibition on relative cytosolic calcium transients (false colored images based on fura lookup table, scale bar = 100 microns). (B) A significant reduction in the relative amplitude of cytosolic calcium (● Initial stimulation and ● Myr-PKCα/β; $p < 0.05$) as well as total calcium (C; $p < 0.05$; measured as sum Δ F/F) accompanied attenuation of LTFE (as in Figure 3). (D) No differences were observed in LTFE induction or cytosolic calcium (E) after increasing stimulus intensity of the glutamatergic afferents to the pacemaker ($p > 0.05$). However, attenuation of LTFE maintenance was still observed (F; $p < 0.001$).

Increasing the strength of stimulation matched LTFE induction values (**Figure 14D**, n.s.), restored the Ca^{2+} signal to baseline levels (**Figure 14E,** n.s.) and still resulted in a significant reduction in LTFE maintenance (**Figure 14F**, $p < 0.001$) supporting our hypothesis that conventional PKC isoforms mediate LTFE by working downstream of NMDAR activation.

LTFE Also Requires the Atypical Isoform PKCζ

Application of an atypical protein kinase C inhibitor (Myr-PKCζ; 10nM) also reduced PMn basal firing rate by an average of 44.6 Hz or 6.7% ± 0.75% (**Figure 15A**, $p < 0.001$). Inhibition of PKCζ reduced LTFE induction and maintenance (**Figures 15B and C**, $p < 0.001$), and reduced the magnitude of PMn calcium transients by 66% (**Figures 15D**; $p < 0.001$). However, increasing the stimulus intensity to the PMn was unable to restore the induction values when compared to the initial stimulation suggesting that Myr-PKCζ antagonist strongly effects neurotransmission.

To address whether PKCζ contributes the maintenance of LTFE, we reduced the current intensity to the PMn for the initial stimulation and matched PMn calcium transients (**Figure 15E**; n.s.) and measured the effects of PKCζ on LTFE induction and LTFE duration. No differences in induction (data not shown) were observed, however, LTFE maintenance was significantly attenuated as compared to the initial stimulation (**Figure 15F**, $p < 0.001$)

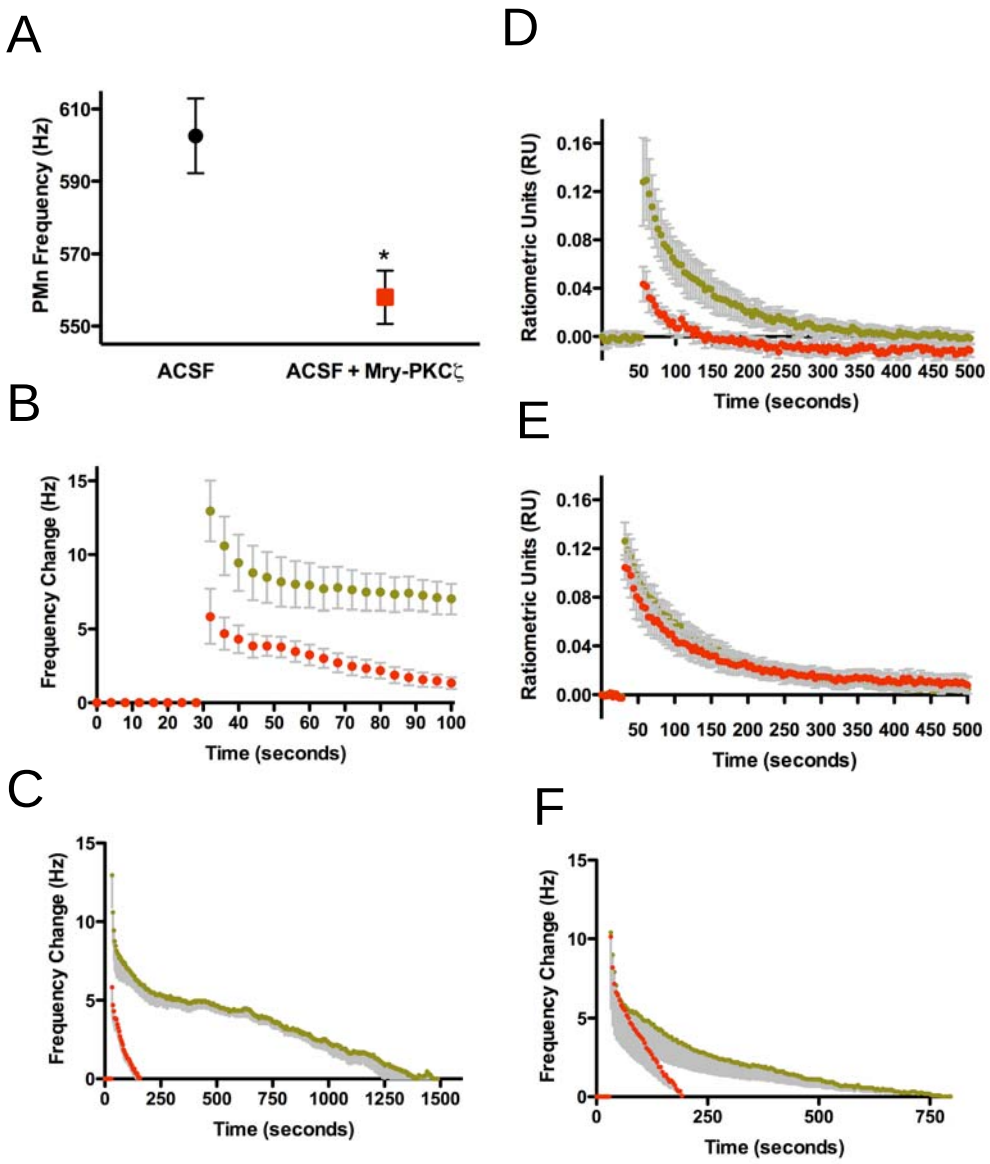


Figure 15: Inhibition of the atypical isoform PKCζ and LTFE.

(A) The application of a specific PKCζ antagonist (myristolated PKC oligopeptide generated against the catalytic domain of PKCζ) reduced PMn basal firing (● ACSF alone, ■ Myr-PKCζ, 10 nM $p < 0.001$). However, increasing the stimulus intensity to the PMn could not reverse the effect of the antagonist on LTFE induction or maintenance (B and C respectively; ● Initial stimulation and ● Myr-PKCζ, $p < 0.001$). (D) The attenuation in LTFE was accompanied by a significant reduction in the relative cytosolic calcium transients in the presence of the PKCζ antagonist ($p < 0.001$). (E) After reducing the stimulus intensity (see methods) of the initial stimulation to match the amount of induction in the presence of the inhibitor, no differences were observed in the relative cytosolic calcium transients ($p > 0.05$) and LTFE maintenance was significantly attenuated (F; $p < 0.001$).

Calcineurin As a Negative Regulator of LTFE

Given that LTFE is established by protein kinases it is logical to assume that protein phosphatases stabilize PMn activity by dephosphorlyating phosphoproteins targeted by kinases and, in turn, act as inhibitory constraints on LTFE. LTFE was elicited in the presence of the calcineurin antagonists, cyclosporin A (cyclo A; 10 uM) and FK506 (3uM). Application of cyclo A had no effect on PMn basal firing (**Figure 16A**, $p > 0.05$). However, LTFE maintenance was enhanced (Fig. 6B, $p < 0.001$) with no change in LTFE induction (**Figure 16B'**, $p > 0.05$).

Although cyclo A is a potent calcineurin antagonist, it is known to influence other enzymes than calcineurin (Jacinto et al., 1998). Therefore, we treated pacemaker slices with another calcineurin antagonist, FK506 (3 uM). Under the same stimulation parameters, the maintenance phase of LTFE was enhanced (**Figure 16D**, $p < 0.001$) with no observable change in induction (**Figure 16D'**, $p > 0.05$). In both experiments, vehicle alone (DMSO 0.1%) had no affect on basal firing rate or on LTFE (data not shown). Imaging calcium transients in the PMn before and after the application of cyclo A demonstrating that inhibiting calcineurin does not alter LTFE induction or PMn calcium signaling (**Figure 17A and B**, n.s.) yet, as previously demonstrated, enhanced the maintenance phase of LTFE (**Figure 17C**, $p < 0.001$).

Because calcineurin can exert its effects through other phosphatases such as PP1 and PP2A, we tested whether these phosphatases play a role in LTFE. The application of okadaic acid (100 uM) did not alter PMn basal firing rate or LTFE induction (**Figures 18A and B, n.s.) .** However, LTFE duration was enhanced (**Figure 18C**, $p < 0.001$)

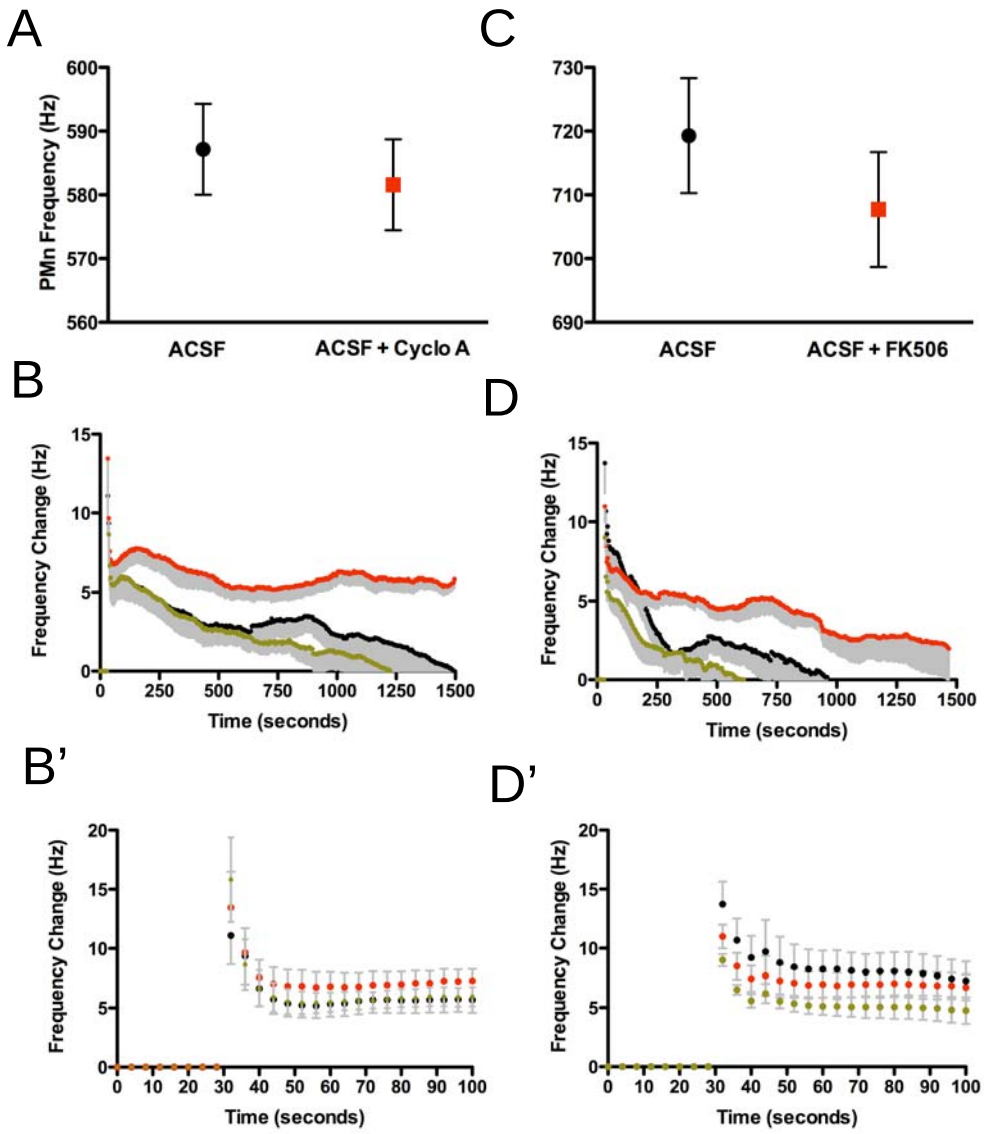


Figure 16: Inhibition of the calmodulin-dependent phosphatase, calcineurin, enhances LTFE.

Bath perfusion of specific calcineurin inhibitors, cyclosporin A and FK506 had no effect on PMn basal firing rate (A and B respectively; ● ACSF alone, ■ cyclosporin A, 10uM; ● ACSF alone, ■ FK506, 3uM; n.s.). (C) However, cyclosporin A enhanced LTFE maintenance (● initial stimulation, ● cyclosporin A, ● wash stimulation; $p < 0.001$) as well as FK506 (D; ● initial stimulation, ● FK506, ● wash stimulation; $p < 0.001$). Cyclosporin A and FK506 did not effect LTFE induction (E and F respectively; n.s.).

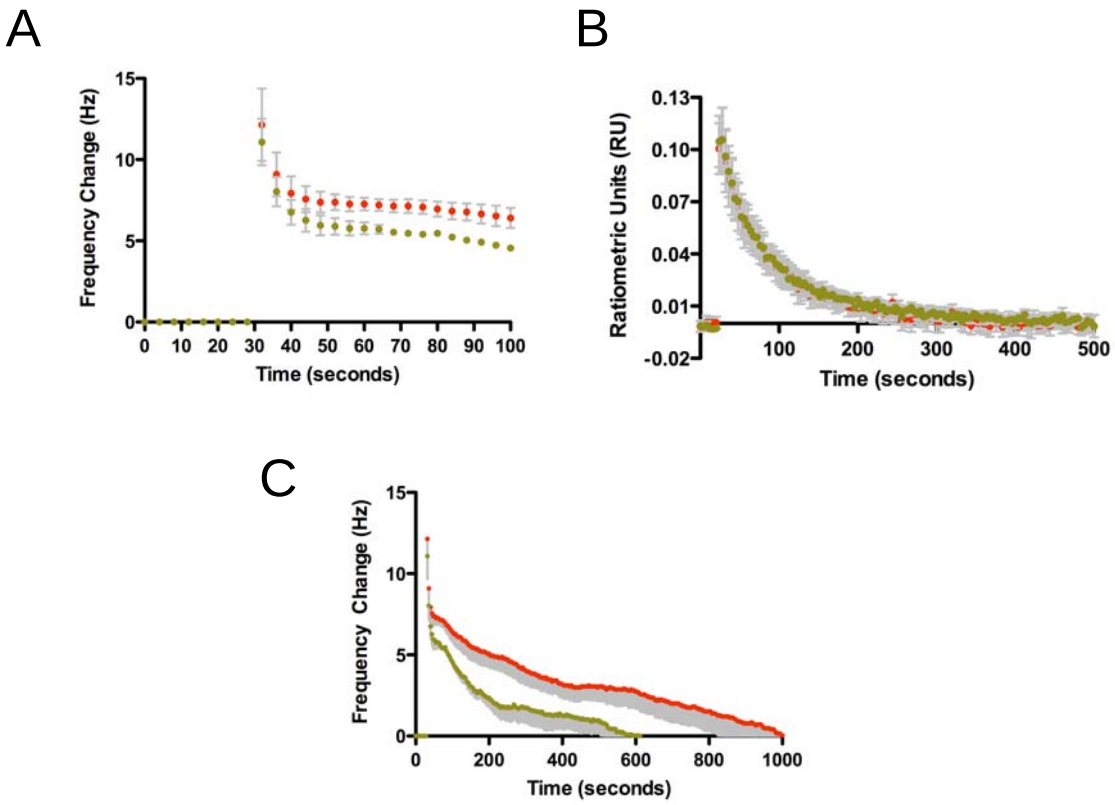


Figure 17: Inhibition of calcineurin has no effect on calcium signaling in the PMn.

The application of cyclosporin A does not affect LTFE induction (A; ● Initial stimulation and ● cyclosporin A, 10uM; n.s.) or relative cytosolic calcium after LTFE induction (B; n.s.). Calcineurin inhibition still enhances the maintenance of LTFE (C; $p < 0.001$).

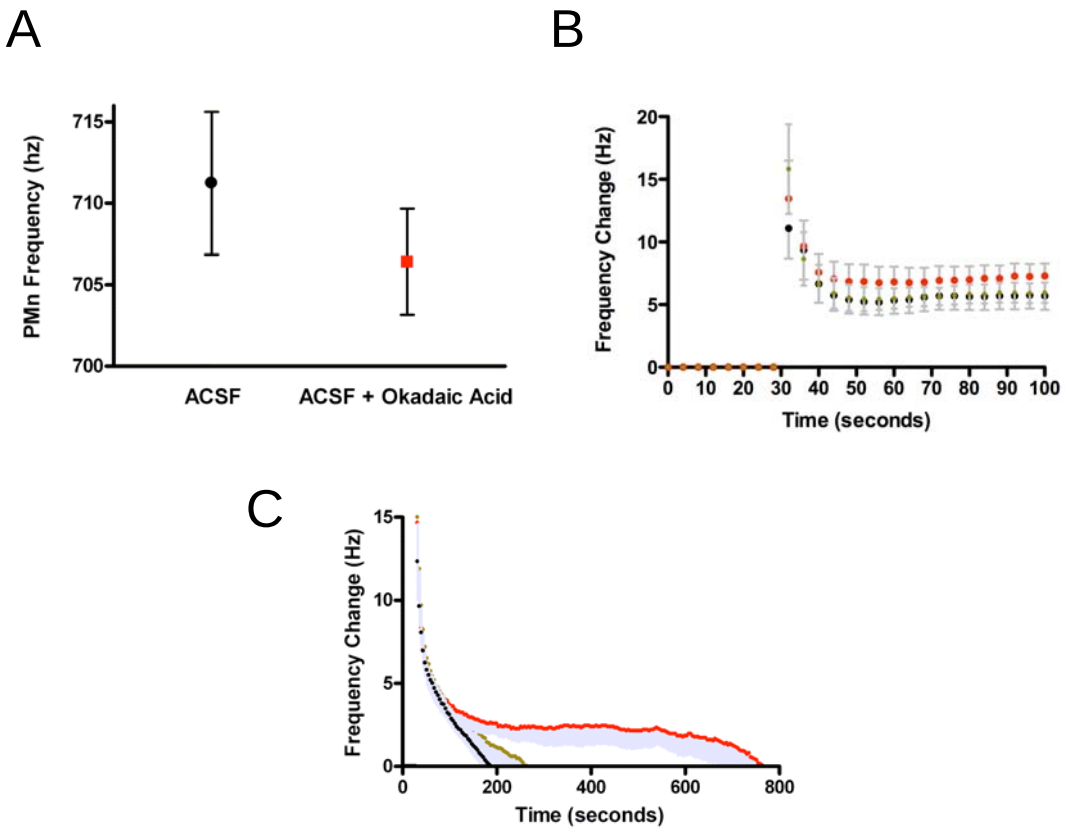


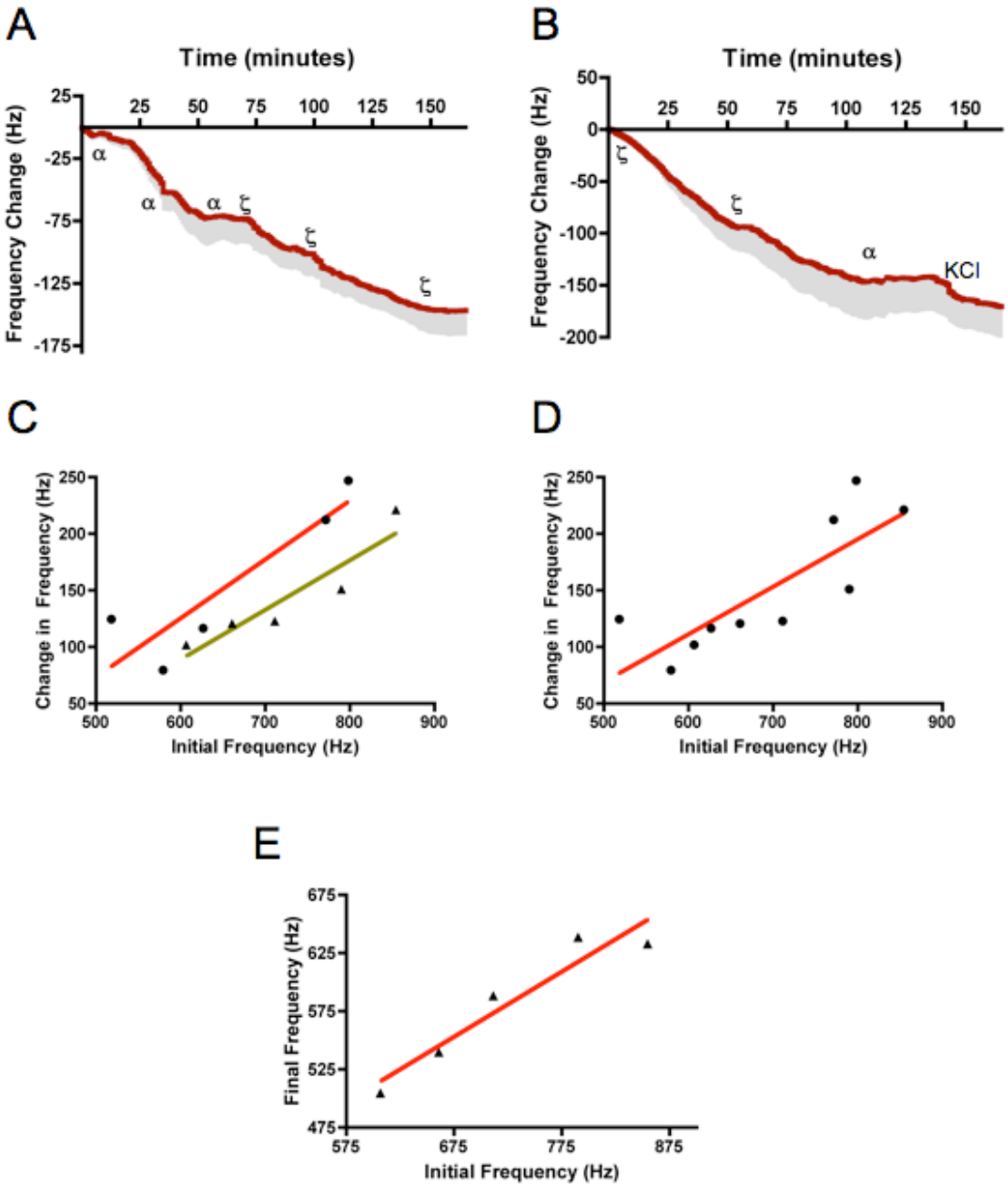
Figure 18: Inhibition of protein phosphatases 1 and 2A (PP1 and PP2A) have similar effects on LTFE.

(A) The application of okadaic acid does not affect PMn basal firing (● ACSF alone, ■ OA 100 nM, n.s.) or LTFE induction (B; n.s.). PP1 and PP2A inhibition still enhances the maintenance of LTFE (C; ● initial stimulation, ● OA ● wash stimulation; $p < 0.001$).

Conventional and Atypical PKC Isoforms and PMn Spontaneous Activity

Since inhibition of both conventional and atypical forms of PKC reduced PMn basal firing rate we tested whether the effects of blocking these two kinases were additive. We initially treated pacemaker nuclei with increasing concentrations of the conventional PKC antagonist to identify a saturating concentration (**Figure 19A**; Myr-PKCα/β; 40 nM). PMn firing frequency decreased 72.4 Hz ± 4.5 Hz and no further decrease in firing frequency was observed with higher concentrations (**Figure 19A**; time point: 55 minutes). After ~ 15 minutes of stable activity, we added the atypical antagonist to the bath in the continued presence of the PKCα/β antagonist (**Figure 19A**; time point: 70 minutes), increasing the concentration until saturation and a final decrement in firing rate of 147.5 Hz ± 3.3 Hz (**Figure 19A**; time point: 158 minutes).

Surprisingly, reversing the order of application produced a different result. Superfusing PMn slices with the PKCζ antagonist at a saturating dose (**Figure 19B**; Myr-PKCζ; 40 nM) decreased PMn firing rate 143.7 Hz ± 19.5 Hz. Subsequent application of PKCα/β antagonist (**Figure 18B**; time point: 120 minutes) had no further effect on firing rate. To ensure that the inability of PKCα/β blockers to further lower firing rate was not an artifact of reaching some intrinsic lowest limit of PMn firing rate, we reduced the external KCl concentration (**Figure 19B**, 2mM to 1mM; time point: 140 minutes). This resulted in further reduction of PMn basal firing rate by 23.8 Hz ± 8.5Hz. These results show that the interaction of the two kinases is complex and non-additive and that blocking atypical kinase completely occludes the effects of blocking of conventional PKC isoforms.


A
Time (minutes)
Frequency Change (Hz)
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-25
-75
-125
-175
25
50
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Time (minutes)
Frequency Change (Hz)
50
0
-50
-100
-150
-200
25
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75
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675
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525
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575
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Initial Frequency (Hz)

Figure 19: Interaction between conventional and atypical PKC isoforms in regulating PMn basal activity.

(A) PMn slices were exposed to increasing concentrations of the conventional PKC antagonist (Myr-PKCα/β; each α symbol representing an increase in concentration) until a saturating dose did not produce any further decrement in PMn basal firing (40nM). After 20 minutes of stable activity, bath application of the atypical PKC antagonist (A; Myr-PKCζ; each ζ represents an increase in concentration) further reduced PMn basal firing rate (saturating dose of PKCζ at 40 nM). (B) Reversing the order of PKC inhibition resulted in the occlusion of the effects of PKCα by the effects of PKCζ. Reducing the concentration of external potassium (from 2mM to 1mM KCl) further reduced PMn firing rate (time point 140 minutes). (C) For both sets of experiments, the change in PMn firing rate positively correlated with initial firing frequency when the alpha inhibitor was applied first (green line, $p < 0.05$) or when the PKCζ inhibitor was applied first (red line, $p < 0.05$). (D) Regression analysis also showed a significant correlation when combining the two data sets ($p < 0.05$). (E) A significant correlation still existed when plotting the final frequency as a function of the initial PMn frequency ($p < 0.05$).

Regression analysis showed a significant correlation between the magnitude of reduction in spontaneous firing and the original PMn original firing frequency (i.e. the higher the initial frequency, the greater the change in firing rate) for experiments where inhibition of PKCα/β occurred before the inhibition of PKCζ (**Figure 19C**, $p < 0.05$, $r^2 = 0.8068$, $F = 12.53$, slope = 0.5198 ± 0.1469) and those where inhibition of PKCζ occurred before the inhibition of PKCα/β (**Figure 19C**, $p < 0.05$, $r^2 = 0.8601$, $F = 18.45$, slope = 0.4382 ± 0.1020). Regression analysis also showed a significant correlation when combining both data sets (**Figure 19D**, $p < 0.05$, $r^2 = 0.6663$, $F = 15.97$, slope = 0.4203 ± 0.1052). A positive correlation also existed when determining the final frequency as a function of original frequency (**Figure 19E**, $p < 0.05$, $r^2 = 0.9101$, $F = 30.36$, slope = 0.5608 ± 0.1018). Controlling for the change in PMn firing rate and holding the final frequency as a constant, multiple regression analysis demonstrated that PKCs contribute to ~ 33% of the total variation.

DISCUSSION

These findings demonstrate that a novel form of synaptically-induced neural plasticity, long-term frequency elevation (LTFE) in the pacemaker nucleus of *Apteronotus leptorhynchus,* is regulated by calcium-dependant phosphorylation. I demonstrate that CaMKII is not involved in LTFE and that inhibition of conventional and atypical PKCs attenuates both LTFE induction and maintenance. Conversely, calmodulin and the calmodulin-activated phosphatase, calcineurin, regulate the duration of LTFE. By simultaneously imaging PMn calcium transients during the inhibition of PKC and calcineurin I demonstrate that these effectors do not alter calcium signaling when LTFE induction is matched and that the effects of PKC and calcineurin work downstream of NMDAR activation. In addition to the mechanisms that regulate LTFE, I also demonstrate that conventional and atypical PKCs interact non-additively to regulate PMn spontaneous activity.

CaMKII Does Not Contribute to Long Lasting Changes in PMn Excitability

CaMKII has long been described as a biochemical switch that converts increases in intracellular Ca^{2+} into long lasting changes in synaptic strength (Hudmon and Schulman, 2002; Lisman et al., 1997; Lisman et al., 2002) or intrinsic excitability (Nelson et al., 2005; Smith et al., 2002). Blocking CaMKII with KN-62 did not affect PMn basal firing rate or LTFE. On the other hand, CaMKII at a lower concentration (3.5 uM) blocks post-tetanic potentiation in other brain regions of this species (Wang and Maler, 1998). In accordance with this, CaMKII is absent in the PMn but is highly expressed in the areas of the *Apteronotid* brain that show post-tetanic potentiation (Maler

and Hincke, 1999). Furthermore, since CaMKII is activated by calmodulin, inhibiting calmodulin should reduce LTFE if CaMKII initiates LTFE. Paradoxically, inhibiting calmodulin enhanced LTFE maintenance, an effect I believe is due to CaM activating the phosphatase calcineurin (see below).

Conventional and Atypical PKCs Contribute to LTFE Induction and Maintenance

Blocking conventional PKC isoforms significantly attenuates the induction of LTFE, the magnitude of the PMn calcium transients as well as the maintenance of LTFE. LTFE induction and calcium signaling can be restored to pre-drug levels by increasing the stimulus intensity to the presynaptic fibers. The restoration of induction is consistent with previous reports that blocking conventional PKCs reduces the probability of neurotransmitter release (Herlitze et al., 2001; Malenka et al., 1986; Suh et al., 2008) and has been demonstrated in many systems (Blackwell, 2006; Torres et al., 2002; Yawo, 1999; Zamponi et al., 1997).

Ca^{2+} transients in the pacemaker were no different from control after matching the induction demonstrating that inhibition of PKC does not alter NMDAR Ca^{2+} permeability, aspects of the Ca^{2+} regulatory process (e.g. Ca^{2+} pumps) that are not involved in LTFE, nor act ectopically on intracellular sources of Ca^{2+} (Banke et al., 2000; Chen et al., 2004; Tong et al., 1995; Yakel, 1997). Nevertheless, after matching LTFE induction and magnitude of the calcium signaling, we still observed a significant attenuation in LTFE maintenance.

A constitutively active form of an atypical protein kinase C isozyme, protein kinase M zeta (PKMζ), is necessary and sufficient for LTP maintenance (Hrabetova and Sacktor, 1996; Yao et al., 2008). We found that the application of a specific PKCζ

antagonist also significantly reduced LTFE induction, maintenance, and magnitude of the calcium signal. Increasing the stimulus intensity to the PMn could not overcome the effect of PKCζ inhibition. Blocking PKCζ in the photoreceptors of mice decreases neurotransmitter release by inhibiting L-type presynaptic calcium channels (Lee et al., 2007). Our data suggest a similar effect in the presynaptic terminals of afferents to the PMn, with the exception that prepacemaker afferents express N-type Ca^{2+} channels (Oestreich et al., 2006). Our study and Lee et al., (2007) emphasize that PKCζ may be a general regulator of neurotransmitter release in different cells types.

To test whether PKCζ also plays a role in LTFE, we decreased the intensity of the initial stimulation (see methods) to allow us to match LTFE induction before and after drug application and observed that the PMn Ca^{2+} transients were not significantly different but that the significant decrease in LTFE maintenance remained. The ability to match PMn Ca^{2+}transients while still attenuating LTFE maintenance suggest that the increase in firing rate is not due to regulation of Ca^{2+} but rather other cationic currents.

At this time it is unclear whether an interaction between conventional and atypical PKCs plays a role in LTFE. It would be nearly impossible to determine an interaction because the stimulus paradigm would require matching LTFE induction values by increasing the stimulus presented in the presence of the PKCα/β antagonist and subsequently trying to match LTFE induction by decreasing the stimulus amplitude in the presence of the PKCζ antagonist. Because the inhibition of PKCζ cannot be overcome by increasing the stimulus intensity (see results) we would not know whether the effect was synergistic or simply due to the effect of PKCζ inhibition alone. However, we assume they interact during LTFE in the same way that they do in regulating basal firing rate (see below).

Calcineurin is Necessary in Regulating the Duration of LTFE

Calcineurin inhibition significantly enhanced LTFE maintenance with no effect on PMn spontaneous activity. In the present study, calcineurin inhibition did not alter LTFE induction or the PMN Ca^{2+} transients. These data suggests that the inhibition of calcineurin does not alter neurotransmitter release, NMDAR function, or any other postsynaptic source of Ca^{2+}. Inhibition of calmodulin also enhanced the maintenance of LTFE with no effect on LTFE induction. This is not surprising because the activation of calcineurin is dependent on calmodulin and these data support the findings that calcineurin plays an influential role in signaling pathways that are recruited for synaptically induced plasticity (Malleret et al., 2001).

Calcineurin acts via a cascade of phosphatases including PP1, and PP2A (Halpain et al., 1990). Inhibition of these phosphatases with okadaic acid produced the same results as inhibition of calcineurin in the PMn (data not shown). Similar to our findings, calcineurin does not regulate basal electrical activity of hippocampal pyramidal cells but is instrumental is mediating synaptically-induced Ca^{2+}-dependent decreases in excitability (Misonou et al., 2005; Misonou et al., 2004; Mohapatra et al., 2007; Surmeier and Foehring, 2004). Together these data indicate that calcineurin may be recruited during synaptically-induced intrinsic plasticity in numerous cell types to act as a constraint on phosphorylation-dependent increases in firing rate.

PMn Spontaneous Activity is Regulated by the Conventional and Atypical PKCs

The application of PKCα/β and PKCζ inhibitors independently reduced PMn spontaneous firing. Previous studies have demonstrated that PKCs influence bursting and other prolonged changes in neuronal activity including spontaneous activity (Magoski, 2004; Wang et al., 1997; Wilson et al., 1998) by modulating the activity of many ion channels directly (Chen et al., 2006; Desai et al., 2008; Numann et al., 1991). The PMn of *A. leptorhynchus* is regulated by at least four cationic currents (Oestreich et al., 2006; Smith, 2006) and it will be important to determine in future experiments whether the channels that regulate PMn intrinsic firing are targeted by PKCα/β and PKCζ.

Because both PKC inhibitors independently reduced firing rate, we asked whether the two kinases worked in parallel or in series. We initially applied the PKCα/β antagonist until saturating concentrations produced no further decrement in PMn firing (~75Hz). The subsequent application of the PKCζ antagonist further reduced PMn firing until saturating concentrations produced no further effect (average reduction ~150Hz) indicating that the effects of inhibiting PKCα/β are additive to the effects of inhibiting PKCζ. By reversing the order, saturating doses of the PKCζ antagonist alone reduced the average firing frequency by ~150 Hz and the application of the PKCα/β antagonist had no further effect on reducing PMn firing rate. Because the inhibition of PKCζ occluded any further effects of PKCα/β inhibition it is likely that they work in series. The simplest interpretation of these results is that PKCα/β regulates PKCζ.

Relationship of PKC to Sexual Dimorphism in the EOD Frequency

The EOD of *A. leptorhynchus*, is sexually dimorphic, with females discharging between 600-800 Hz and males between 800-1100 Hz (Hagedorn and Heiligenberg, 1985), and each fish has a unique EOD frequency within the frequency range of its gender (Schaefer and Zakon, 1996). The PMn controls EOD frequency in a 1:1 manner and the firing rate of the PMn in slice is approximately the same as the EOD frequency of the fish from which it came (Meyer et al., 1987b). Inhibition of PKC resulted in a change in PMn firing rate that positively correlated with the initial firing frequency. The maximum drop in firing rate following total blockade of PKC was ~250 Hz. This is comparable to the total range of either sex, or 50% of the total species range. We did not assess the gender of the fish in this study so we cannot directly address the extent to which PKC plays a role in determining EOD frequency within each sex. But since the PMn firing rate of every fish that we tested was affected by PKC block, we believe that PKC exerts a powerful effect on setting baseline EOD frequency in this species.

A comparison of PMn firing rate after PKC inhibition with initial firing frequency, also yielded a strong positive correlation suggesting that PMn firing rate is governed jointly by PKC and additional regulatory mechanisms. One interesting possibility is that an interaction exists between steroid hormones and PKC whereby the basal pacemaker frequency is determined by the effects of steroid hormones while phosphorylation by PKC provides additional tonic frequency enhancement. EOD frequency in this species is shifted over days to weeks by steroid hormones (Zakon and Dunlap, 1999), likely through genomic transcriptional regulation. These hormones could be acting through several mechanisms, such as modulating the levels of one or more ion channels and/or regulating PKC levels.

A Model for Phosphorylation-Dependent Regulation of Firing Rate and LTFE

PMn spontaneous activity is regulated by conventional and atypical PKCs in the absence of synaptic stimulation **(Figure 20)**. Conventional PKCs are activated by diacylglycerol (DAG) and/or Ca^{2+}. It is likely that DAG constitutively activates conventional PKCs in the PMn because blockade of phospholipase C markedly lowers PMn spontaneous firing rate (A. George, unpubl.). In other systems, PKCζ is activated by other kinases, such as PDK1 kinase (Kelly et al., 2007).

We do not know which kinases regulate PKCζ in the PMn although our results suggest a serial interaction between conventional and atypical PKCs where PKCα/β are upstream of PKCζ. However, because blockade of PKCα/β does not occlude the effects of PKCζ inhibitors, PKCζ must be activated by other kinases as well. Finally, because the inhibition of calcineurin, PP1, and PP2A does not affect PMn basal firing rate (A. George, unpubl.), another constitutively active phosphatase must be involved in regulating PMn basal activity. We suppose that this phosphatase is not in high concentration or very efficient as it takes an hour for basal firing rate to decrease to a minimum after total blockade of both PKC isoforms.

With activation of the NMDA receptor and the subsequent Ca^{2+} influx that induces LTFE, we propose that Ca^{2+} boosts the activity of already active conventional PKCs as well as activating calcineurin. When calcineurin is pharmacologically blocked, PMn firing rate remains elevated for many minutes, again, indicating that the endogenous unidentified phosphatase do not act rapidly. Thus, calcineurin is recruited as a brake in the system to determine the duration of LTFE.

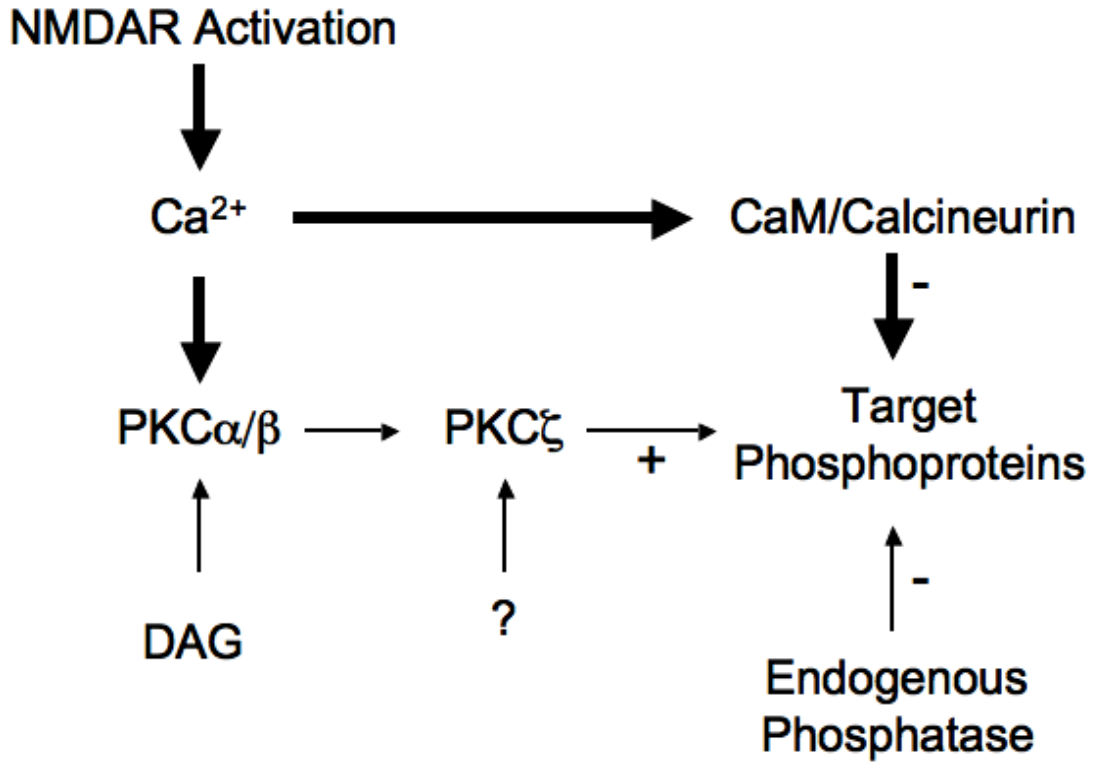


Figure 20: Modeling PMn mechanisms of stability and plasticity.

PMn spontaneous activity is regulated by a serial interaction between conventional and atypical PKCs. During the jamming avoidance response, NMDAR activation leads to an influx of Ca^{2+} biasing the activity of conventional PKC isoforms and ultimately leading to the phosphorylation of target proteins involved in LTFE. The same Ca^{2+} signal activates the calmodulin/calcineurin pathway leading to the dephosphorylation of proteins targeted by PKCs and ultimately, reducing the PMn firing rate to basal levels.

Collectively, these data contribute to understanding the mechanisms that are responsible for maintaining the excitability of entire neural circuits and how these mechanisms restore normal patterns of activity when destabilizing effects, such as transient synaptic stimulation, perturb network activity.

Chapter 4: Species-Specific Differences in Neural Intrinsic Excitability are Mirrored by Differences in Pacemaker Neurochemistry

ABSTACT

In this chapter I demonstrate that the weakly electric fish, *A. leptorhynchus* and *E. virescens*, differentially express conventional and atypical PKCs. I assayed for the expression of three conventional PKC isoforms (PKCα, PKCβ and PKCγ) as well as atypical PKC (PKCζ), in the pacemaker nuclei of *Apteronotus leptorhynchus* and *Eigenmannia virescens.* In *A. leptorhynchus* PMn expression of PKCα is abundant whereas in *E. virescens*, this isoform is nearly absent. Pacemaker expression of PKCβ is equal in both species while PKCγ, an isoform solely expressed in the brain and spinal cord and whose localization is restricted to neurons in mammals (Saito and Shirai, 2002) is absent. Expression of PKCζ, an atypical isoform is expressed in the PMn of both species. In addition, I demonstrate that Ca^{2+}/calmodulin dependent phosphatase, calcineurin (also known as protein phosphatases 2B or PP2B) expression in *A. leptorhynchus* is greater than in *E. virescens.* Together, these data demonstrate that the species-specific differences in sensorimotor adaptation are mirrored by differences in PMn neurochemistry.

INTRODUCTION

While many studies on activity-dependent changes in neuronal function have focused on global long-term changes caused by altered gene expression, less attention has been given to the activity-dependent alterations in the intrinsic electrical properties of

neurons (Misonou et al., 2004). These alterations are extremely dynamic, and often require the coordinated effort of calcium-mediated enzymatic processes. A number of cellular proteins are recruited for this purpose, which themselves are regulated by protein phosphorylation (Groth et al., 2003).

Weakly electric fish generate electric fields around their body through a specialized electric organ and senses these electric organ discharges (EODs) for electrolocation and electrocommunication (Hopkins et al., 1997). Electric fish are nocturnally active and during daylight hours they avoid predators by hiding in thick grasses along river banks (Crampton, 1998; Hagedorn and Heiligenberg, 1985). In the wild, *A. leptorhynchus* are solitary (Hagedorn and Heiligenberg, 1985; Oestreich 2005; Schwassmann, 1978; Zakon, H. H., personal communication) and, in contrast, *E. virescens* are often found in large shoals (Oestreich 2005; Zakon, H. H., personal communication).

In *Apteronotus* and *Eigenmannia* the EOD frequency is individual- and, gender-specific (Hopkins, 1988). However, the probability of being jammed increases with group size and is therefore higher in *Eigenmannia*. One possible solution to escape jamming is to simply swim away from other individuals, because electric field strength falls off with increased distance from the jamming source (Knudsen E.I., 1975). However, leaving the safety of the shoal could come with the cost of predation (Lismann, 1961). Recent findings demonstrate that both species adjust their EOD frequency to avoid jamming and the duration of LTFE strongly correlates with the strength and duration of the jamming stimulus. With a strong jamming stimulus *in vivo* (6 hour long stimulus presentation), LTFE in *E. virescens* remained stable for at least 16 h after an upward JAR compared to Apternonotus, which exhibits ~ 2 hours of LTFE under the same stimulus parameters. This demonstrates that a persistent readjustment of the EOD

frequency is positively correlated with LTFE magnitude and social organization (Oestreich and Zakon, 2005) .

The EOD frequency is established by an autonomously firing medullary pacemaker nucleus (PMn) that is comprised primarily of pacemaker neurons and relay neurons, which send their axons out of the PMn to innervate spinal motorneurons. (see chapter 2). Pacemaker and relay cells are coupled by gap junctions and entrain each other. Each action potential from the pacemaker elicits an action potential from the electric organ so EOD frequency is determined in a 1:1 fashion by PMn firing frequency.

Previous work has demonstrated that LTFE is not maintained by continuing synaptic input, but results from a long-term increase in the firing frequency of the postsynaptic PMn neurons indicating a change in their intrinsic excitability (Oestreich et al., 2006). Moreover, LTFE is induced by activation of NMDA receptors (Dye et al., 1989). At the postsynaptic level, the activation of NMDARs and subsequent elevation in internal Ca^{2+} can trigger distinct signaling mechanisms that lead to enhanced synaptic function (Malgaroli et al., 1992).

In addition, a number of studies have established roles for protein phosphorylation in modulation of synaptic efficiency. For example, Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) is highly concentrated at synapses that utilize glutamate as the neurotransmitter and it is believed to be one of the major contributors to the enhanced strength of glutamatergic synapses in CA1 area of hippocampus during long-term potentiation (Derkach, 2003). Other Ca^{2+}-mediated enzymes such as protein kinase C (PKC) can regulate ion channels yielding variable states of activation and inactivation (Misonou et al., 2006; Mohapatra and Trimmer, 2006).

Serine/threonine protein kinases and phosphatases (e.g. CaMKII and calcineurin) participate in a number of cellular processes and Ca^{2+}-dependent signal transduction

pathways in model organisms such as yeast, filamentous fungi, plants, vertebrates, and mammals (Fox and Heitman, 2002; Klusener et al., 2002; Lee and Rudd, 2002; Romeis et al., 2001; Xing et al., 2002). This idea is based in part on the observational data taken from PP2B-knockout mice suggesting that a reduction of calcineurin activity facilitates LTP in vitro and in vivo (Malleret et al., 2001).

Given the well-known importance of serine/threonine kinases and phosphatases in neural plasticity, the purpose of this study is to assay for the expression of three conventional PKC isoforms (PKCα, PKCβ and PKCγ) as well as atypical PKC (PKCζ), in the pacemaker nuclei of *Apteronotus leptorhynchus* and *Eigenmannia virescens*. In addition, I describe the expression patterns of calcineurin, a Ca^{2+}/calmodulin dependent phosphatase. In these experiments, I use western blotting and immunohistochemistry on whole brain and pacemaker protein extracts as well as brain slices from each species. I show that *A. leptorhynchus* and *E. virescens*, the pacemaker nuclei of these two species (PMn) differentially express conventional and atypical PKCs. In addition, I demonstrate that calcineurin expression in *A. leptorhynchus* is twice that of *E. virescens*. Together, these data contribute toward understanding the neural mechanisms that underlie species-specific differences in long-term changes in neural excitability.

MATERIALS AND METHODS

Animals

As previously described, wild-caught individuals of the weakly electric fish *Apteronotus leptorhynchus* were obtained through Segrest Farms (Gibsonton, FL) and housed in community tanks in climate-controlled rooms (stable between 26ºC and 28ºC) and under a 12 hr dark/ light cycle. Fish were fed with frozen brine shrimp every 2 days.

Individuals were allowed to acclimatize for 1 month before being used in experiments with water conductivity held at 800 uS/cm.

Cloning Gymnotiform PKC Isoforms

Total brain and pacemaker RNA was isolated from both species using RNA STAT60 (TELTEST, Friendwood, TX). Isolation of *Apteronotus* PMn RNA was performed by microdesection with a pair of iredectomy scissors. The PMn of *Eigenmannia* lies deep within the caudal medulla, therefore it was necessary to use a mircropunch kit to extract PMn tissue leaving as little marginal tissue as possible. After DNAse treatment (37ºC for 30 minutes) RNA samples were reverse-transcribed (RT-PCR) using Invitrogen's Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and first strand cDNA products were amplified using TAKARA Taq Polymerase (TAKARA, Shiga, Japan). Forward and Reverse primers were based on the highly conserved domains of *Danio rerio* PKC mRNA and consensus sequences from mammalian PKC mRNA (human, rat, and mouse).

PCR Primers

PKC (alpha isoform) forward primers: 5'- AGGAGCCACAAGCTGTCCTA -3' ; : 5'-TGTCCTTCGGTGTGTCTGAG -3'; : 5'- CAGGATGATGACGTGGAGTG -3'; : 5'-CATGTCCTTCGGTGTGTCTG -3'; : 5'- ACACAGGTGATGTTGGCAGA -3'; and reverse primers: 5'-TAGGACAGCTTGTGGCTCCT-3'; 5'-GCATCTCGTACAGGAGCACA-3'; 5'-AGGGATTTGGGGTAGGACAC-3'; 5'-TTGGGTTTGAAAGGAGGTTG -3'.

PKC (beta isoform) forward primers: 5'- GATCTGGGACTGGGACTTGA -3'; 5'-CTAAAATCGGTCCCAGCAAA -3'; 5'- GACAATGGACCGCCTGTACT-3'; and reverse primers: 5'-GTACAGGCGGTCCATTGTCT-3'; 5'-AGTACAGGCGGTCCATTGTC-3'; 5'-AAAGTACAGGCGGTCCATTG-3'; 5'-CCACAGAAGGTCTTGGTGGT -3'; 5'- GAAGCCATGTTCCCTGATGT -3'.

PKC (zeta isoform) forward primers: 5'- TCAGAGGACCTGAAGGCAGT -3'; 5'-ATGTGCAAGGAGGGAATCAG-3'; 5'- GAGGGAATCAGACCAGGTGA -3'; and reverse primers: 5'-CTGATTCCCTCCTTGCACAT-3'; 5'-ACCTGGTCTGATTCCCTCCT -3'; 5'-CGGTTTCTTCAGTGGAGAGC -3'.

Samples were resolved on 1% agarose and one product, with the expected size (~500 bp), was gel purified and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Sequences were obtained through the University of Texas at Austin's Institute for Cellular and Molecular Biology Core Facility (Austin, TX) and aligned and analyzed using MacVector software and ClustalW (v1.4) multiple sequence alignment (MacVector Inc., Cary, NC).

Western Blot Analysis

Total brain and PMn protein extracts were made using tissue extraction buffer (T-PER; Pierce biotechnology (Rockford, IL). Samples were prepared with lane marker sample reducing buffer (Pierce Biotechnologies, Rockford, IL) boiled (5 minutes) and cooled at room temperature. Protein samples were run on denaturing precast protein gels (8-16%, 80-100 volts; Precise Protein Gels Pierce, Rockford, IL) and transferred to a

0.45micrometer nitrocellulose membrane (Fisher Scientific, Pittsburgh, PA) using a GENIE BLOTTER (Idea Scientific, Minneapolis, MN). Blots were washed in 1X TBS and blocked with blotto solution (3 % Blotto Solution: 1.5 g milk, 5ml 10X TBS, up to 49 ml H20, shake 1-2 minutes, 1 ml 10% Tween 20, mix and store at 4ºC) for 1 hour at 22ºC.

Incubation with primary antibodies (overnight at 4ºC) was followed by washes (washed in 1X TBS w/ 0.1% Tween-20; 3X at 22ºC; 5 minutes each wash). Blots were then incubated with secondary goat anti-rabbit antibodies conjugated to HRP (1:20,000 dilution; Jackson Immunolabs; 1 hour at 22ºC). Subsequent washing was performed in 1X TBS w/ 0,1% Tween-20 (3X at 22ºC; 5 minutes each wash). Detection was performed with super signal chemiluminescent substrates (Pierce, Rockford, IL) and exposed to Kodak Blue film (Emeryville CA). (abcam Inc., Cambridge, MA). Primary antibodies (rabbit polyclonal IgGs) for PKCα, PKCβ, PKCγ, and PKCζ were obtained from Santa Cruz Biotechnology, Inc. and epitope mapped to the C-terminus of PKCα of human origin (dilutions ranging from 1:200 to 1:1000). Primary antibodies against α-tubulin and calcineurin (PP2B) were obtained form Abcam Inc., Cambridge, MA (dilution ranging from 1:1000 to 1:5000).

Immunohistochemistry

Control and treatment groups were prepared using well-established protein localization protocols (Peng et al., 2005). In brief, PMn were cryosectioned and mounted on subbed and poly-L-lysine coated slides. Sections from both species were mounted on different slides and processed simultaneously. Sections were rinsed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min, and rinsed again before blocking for 20 min with 2% Blotto solution (1X TBS, 2% bovine serum albumin

(BSA) or dried milk, and 0.2% Tween-20). Sections were incubated with rabbit monoclonal antibodies against specific antigens of interest (RBI-Sigma, St. Louis, MO) overnight at 4oC in blocking solution with primary antibody omission as negative controls.

Sections were rinsed again and incubated for 30 min with 2% Blotto solution containing biotinylated secondary antibody (goat anti-rabbit IgG; Vector Labs, Burlingame, CA), then avidin-Alexa 488 (Molecular Probes, Eugene, OR), with PBS rinses between steps. Immunofluorescent images were visualized using an Olympus IX70 Inverted Microscope and Image Pro Plus v. 4.1 software (Silver Spring, Maryland).

Statistical Analysis

All statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software Inc., San Diego CA). Means and standard error of the mean were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Graphpad software determined significance for unpaired Student's t test (significance determined as $p < 0.05$).

RESULTS

Expression of PKC isoforms in *A. leptorhynchus* and *E. virescens*

To identify PKC isoforms that may contribute to PMn spontaneous activity total brain RNA was harvested from *A. leptorhynchus* and *E. virescens* and the expression patterns of conventional and atypical PKCs were determined by RT-PCR (see methods). Partial sequences obtained from *A. leptorhynchus* and *E. virescens* demonstrated the

presence of PKCα in total brain extracts for both species when aligned with human, rat, mouse and zebrafish known PKCα transcripts (**Figure 21A**).

Identity scores between *A. leptorhynchus* partially cloned sequences and PKCα transcripts for *Homo sapiens, Rattus norvegicus, Mus musculus* and *Danio rerio* were (in %) 72.7, 76.6, 77.8 and 76.6 respectively. Identity scores between *E. virescens* partially cloned sequences and PKCβ transcripts for *Homo sapiens, Rattus norvegicus, Mus musculus* and *Danio rerio* were (in %) 72.9, 76.8, 78.0 and 76.8 respectively (**Figure 21A**).

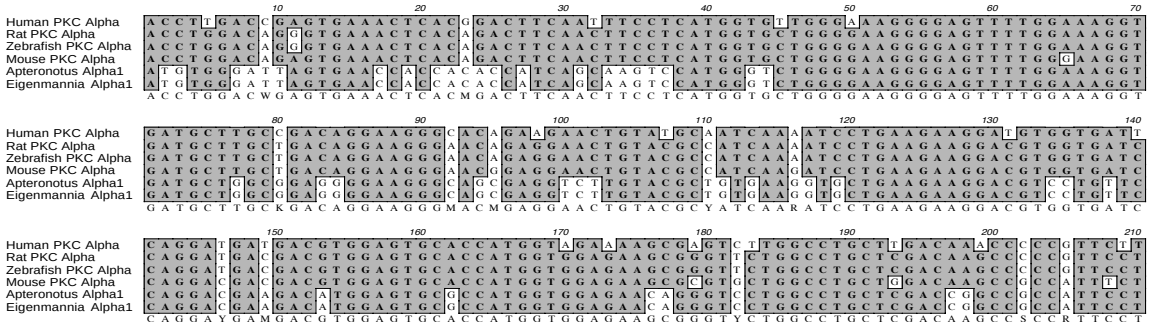
Comparing identity scores between *A. leptorhynchus* partially cloned sequences and PKCβ transcripts for *Homo sapiens, Rattus norvegicus, Mus musculus* and *Danio rerio* revealed identities of (in %) 73.9, 76.8, 77.4 and 86.4 respectively. Identity scores between *E. virescens* partially cloned sequences and PKCβ transcripts for *Homo sapiens, Rattus norvegicus, Mus musculus* and *Danio rerio* were (in %) 78.0, 79.4, 80.3 and 87.5 respectively (**Figure 21B**).

For the atypical isoform, PKCζ, identity scores between *A. leptorhynchus* partially cloned sequences and PKCζ transcripts for *Homo sapiens, Rattus norvegicus, Mus musculus* and *Danio rerio* revealed identities of (in %) 78.5, 78.1, 77.9 and 81.5 respectively. For *E. virescens*, scores between *E. virescens* partially cloned sequences and PKCζ transcripts for *Homo sapiens, Rattus norvegicus, Mus musculus* and *Danio rerio* were (in %) 79.7, 79.7, 78.7 and 79.9 respectively (**Figure 22**). Topology of the gene tree was constructed out of amino acid residues and constrained to preserve the organization of each PKC family. Topology of the PKC genes cloned from *Apteronotus* and *Eigenmannia* revealed homologous identified genes for PKCγ, PKCβ and PKCζ (**Figure 23**). Given the topology of PKC genes and the grouping of PKCa with known

PKCγ sequences, these results indicate that the identified sequence is PKCγ and not PKCα as originally described.

A

PKCα Alignment



B

PKCβ Alignment

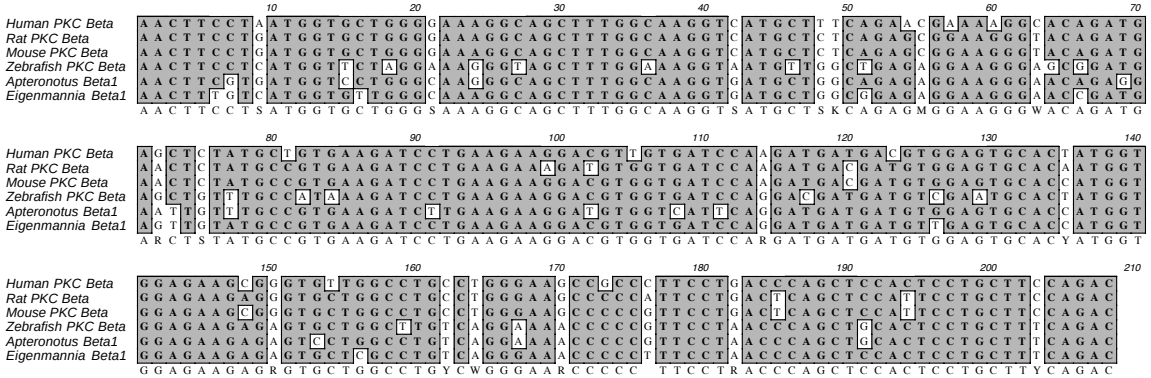


Figure 21: Nucleotide alignments of PKCα and PKCβ isoforms

(A) PKCα and (B) PKCβ mRNA sequences were obtained from the NCBI nucleotide database query and sequences from *Homo sapien, Rattus norvegicus*, *Mus musculus*, *Danio rerio*, were aligned with sequences from *Apteronotus leptorhynchus and Eigenmannia virescens.* Sequence similarities are shaded in grey with a consensus sequence annotated below the alignment.

PKCζ Alignment

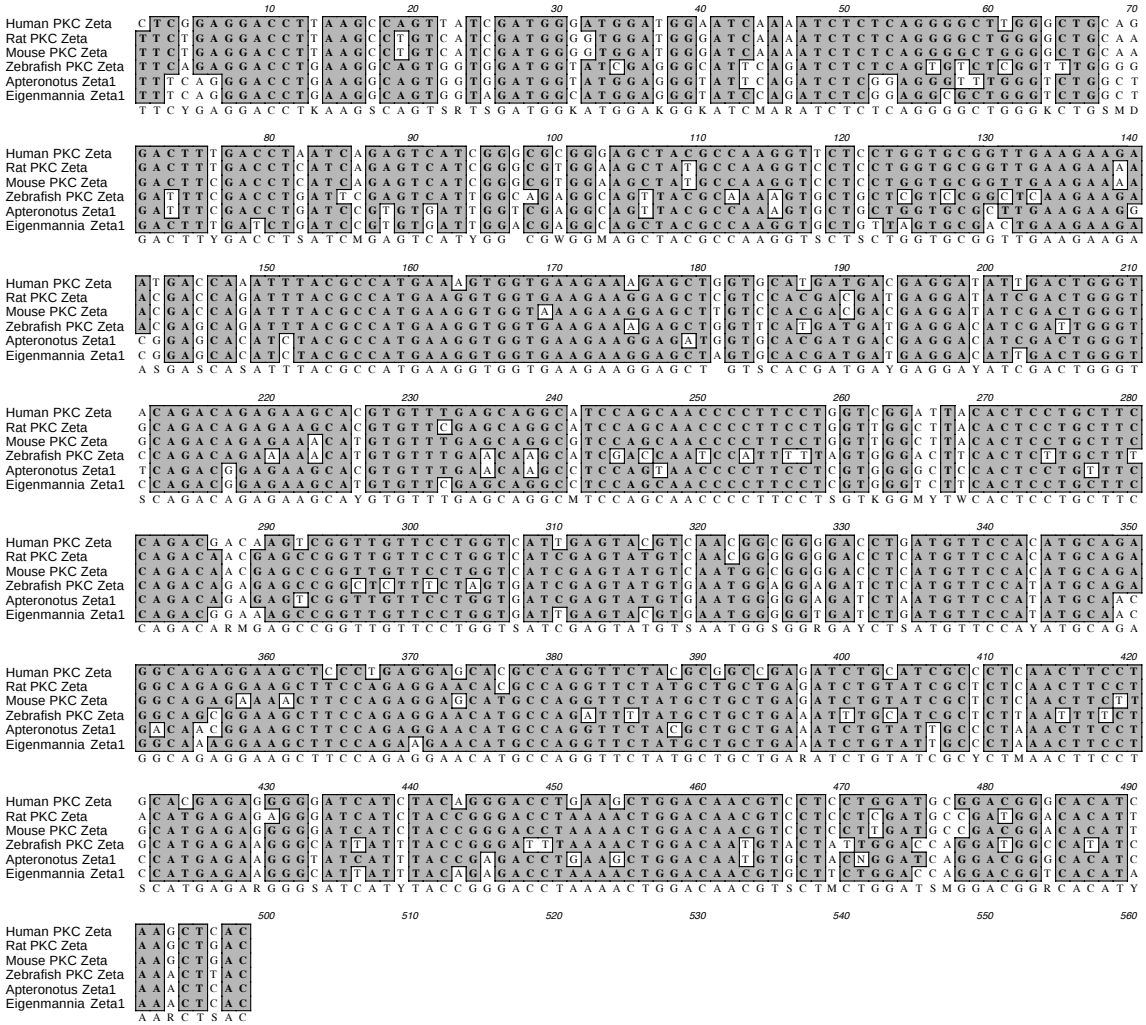


Figure 22: Nucleotide alignment of PKC zeta (PKCζ) isoform

PKC zeta mRNA sequences were obtained from the NCBI nucleotide database query and sequences from *Homo sapien*, *Rattus norvegicus*, *Mus musculus*, *Danio rerio*, were aligned with sequences from *Apteronotus leptorhynchus and Eigenmannia virescens*. Sequence similarities are shaded in grey with a consensus sequence annotated below the alignment.

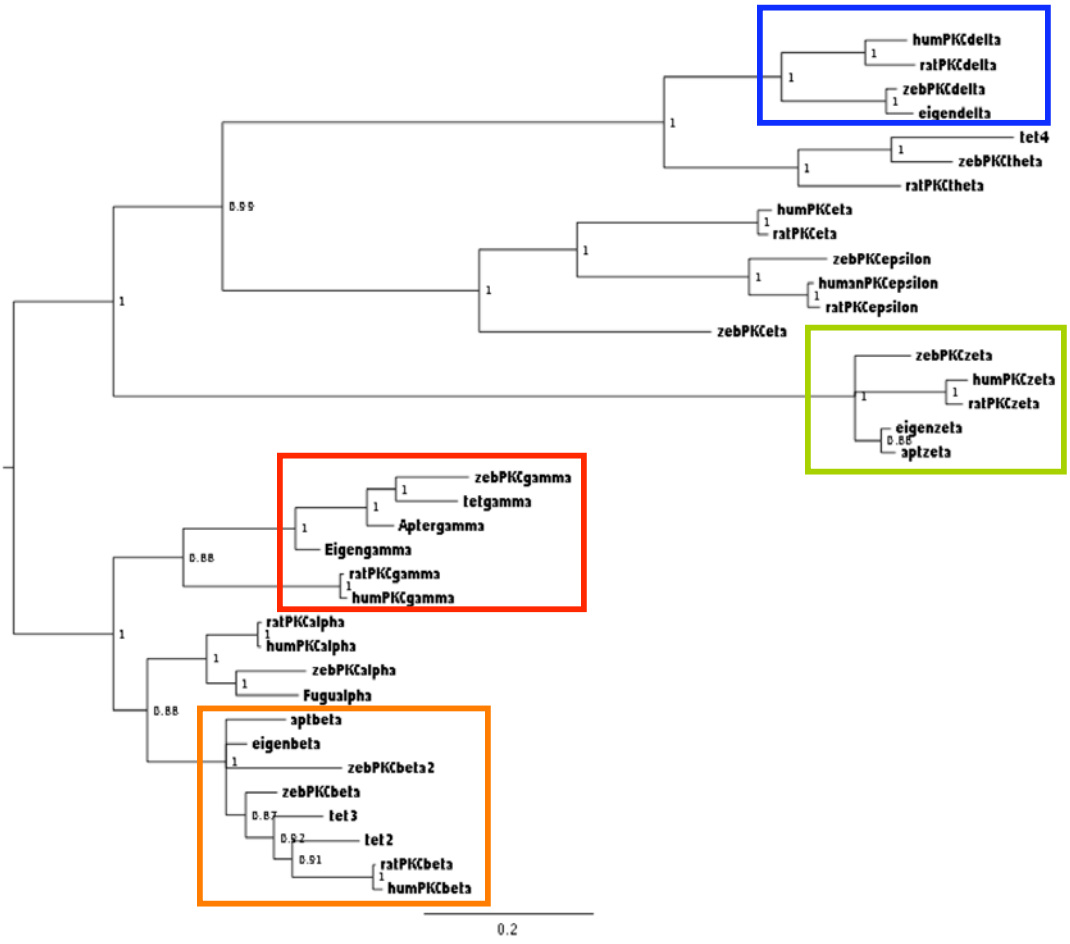


Figure 23: Cladogram of the amino acid sequence of cloned gymnotiform, zebrafish and mammalian PKC families.

Sequences were aligned using Bayesian maximum likelihood methods (values at each node representing posterior probability scores) strongly support PKCγ, PKCβ, and PKCζ as being an ortholog of the mammalian and zebrafish PKCs. Boxes represent PKC groupings (red: PKCγ; orange: PKCβ; green: PKCζ; blue: PKCδ;).

To determine the expression of translated PKC isoforms, total and PMn protein extracts were harvested from *A. leptorhynchus* and *E. virescens* and ran on SDS-polyacrylamide gel electrophoresis and blotted with specific polyclonal antibodies affinity purified and raised against a peptide mapping at the C-terminus of PKCα, PKCβ and PKCγ of human origin (see methods). Bands corresponding to ~80 KDa for the conventional isoforms PKCα, PKCβ and PKCγ were identified from total brain extracts for both species (**Figure 24A**). PMn extracts revealed protein products for PKCα and PKCβ for both species (**Figure 24B**). Conversely, no bands were observed for PKCγ for either *A. leptorhynchus* or *E. virescens* (**Figure 24B**).

Using a polyclonal affinity purified antibody raised against a peptide mapping at the C-terminus of PKCζ of rat origin (see methods), total protein extracts revealed a single protein species corresponding to ~80KDa (**Figure 24A**) for both *A. leptorhynchus* and *E. virescens*. PKCζ expression was also observed in PMn extracts. As an internal control, total brain extracts were made from rat (*Rattus norvegicus*) and run simultaneously against each antibody (**Figures 24A and B**). As a loading control, a specific antibody against α-tubulin was used for both total and PMn extracts. Immunoblotting revealed similar levels of α -tubulin in extracts taken from total and PMn (**Figure 24C**).

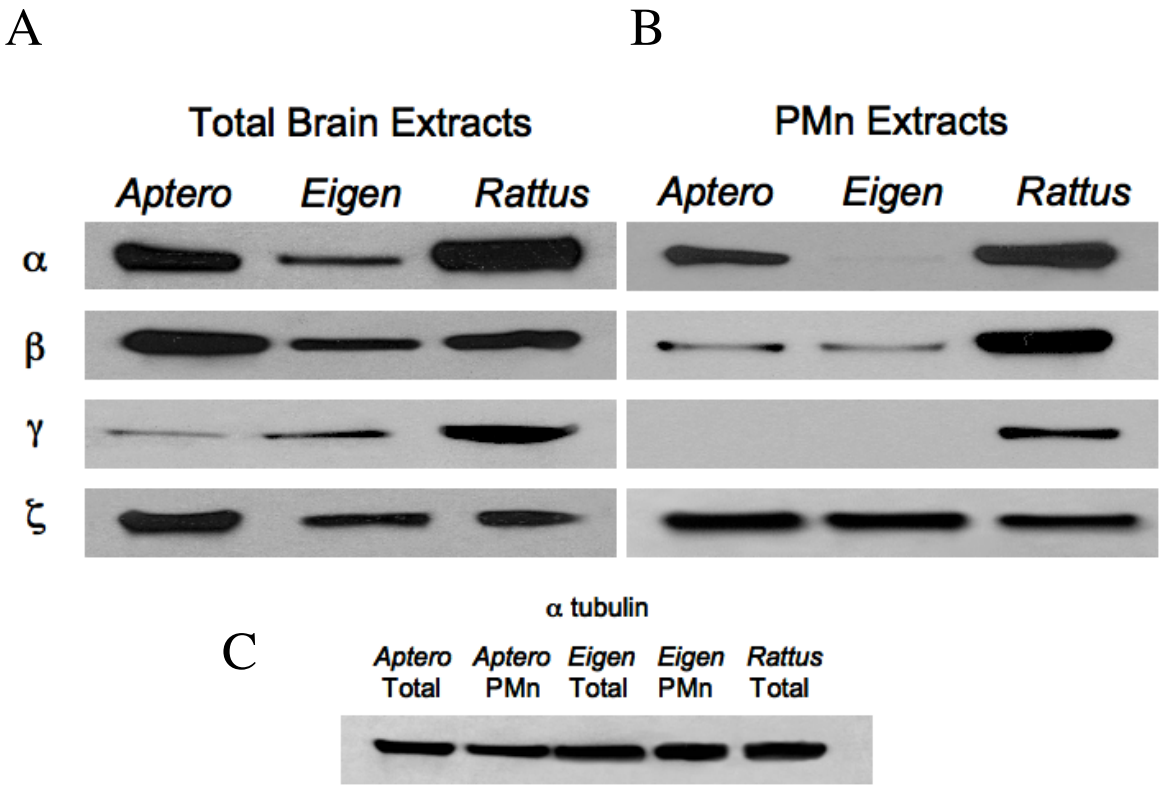


Figure 24: Protein kinase C (PKC) expression in weakly electric fish

Total and PMn protein extracts were run on SDS-page gel electrophoresis and primary antibodies against conventional PKCs (α, β and γ) and atypical PKCs (PKCζ) were used to identify protein expression between *A. leptorhynchus* and *E. virescens*. (A) total brain extracts revealed bands corresponding to ~ 80 KDa for both conventional and atypical PKCs. (B) PMn extracts using the same antibodies. (C) α-tubulin immunoblot loading control. Rat (*Rattus norvegicus*) brain extracts were used as additional internal controls.

Expression of Calcineurin (PP2B) in *A. leptorhynchus* and *E. virescens*

To determine the expression of calcineurin, total and PMn protein extracts were harvested from *A. leptorhynchus* and *E. virescens* and run on SDS-polyacrylamide gel electrophoresis and blotted with specific antibodies against the regulatory domain of calcineurin. Bands corresponding to ~61 KDa were identified from total brain and PMn extracts for both species (**Figure 25A and B**). The PMn in *E. virescens* in embedded deep within the brainstem, hindbrain extracts were prepared to compare the brainstem calcineurin expression vs. PMn micro punches (see methods). In *A. leptorhynchus*, the PMn is located on the ventral surface of the brainstem and is easily excised from the brainstem.

To determine the cell types expressing calcineurin, serial sections from *A. leptorhynchus* and *E. virescens* were incubated with or without a specific antibody against calcineurin. Calcineurin was widely expressed in the PMn of *A. leptorhynchus* (**Figure 26**; a-c, N=3) with immunofluorescence detected in both pacemaker and relay neurons (**Figure 26**. a-c; arrows and arrowheads respectively). Similarly, calcineurin immunofluorescence was observed in pacemaker and relay neurons of *E. virescens* (**Figure 26**; a'-c', N=3).

Calcineurin immunofluorescence was quantified by averaging fluorescence intensities from pacemaker and relay neurons (**Figure 27C**; solid green circles) and measured against unlabeled regions (**Figure 27 C and C'**; dotted green circles). Group data form calcineurin immunohistochemistry showed differences in fluorescence intensities (measured as pixels/um^2) when compared to regions showing little or no calcineurin expression (**Figure 27E**; $p < 0.05$).

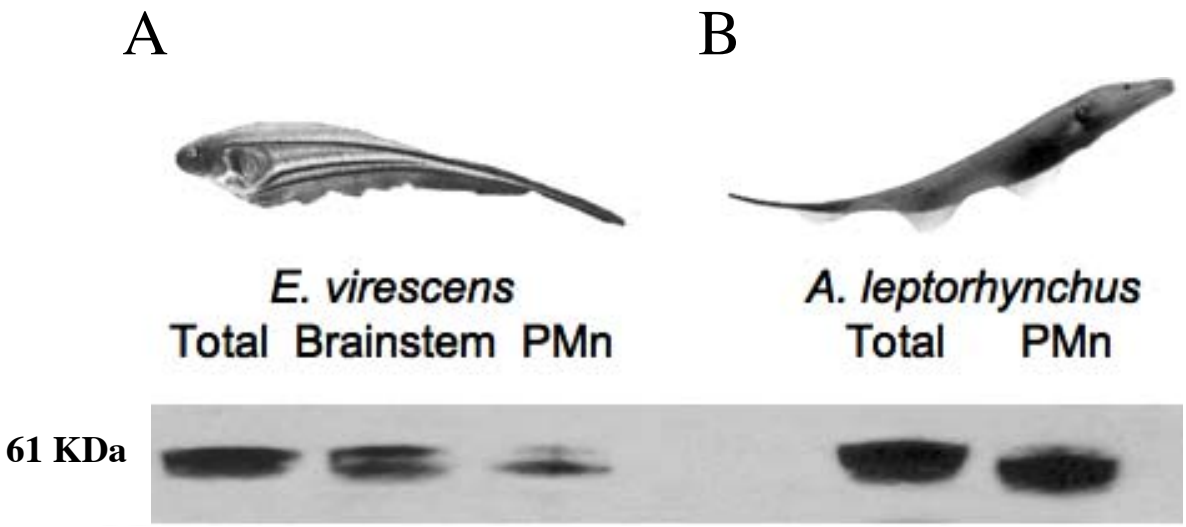


Figure 25: Calcineurin (PP2B) is expressed in shoaling and solitary gymnotiforms

Total and PMn protein extracts were separated on SDS-page gel electrophoresis and an antibody against calcineurin (PP2B) was used to identify protein expression between *E. virescens* and *A. leptorhynchus* (A and B respectively). Total brain and pacemaker extracts revealed bands corresponding to ~ 61 KDa for both species.

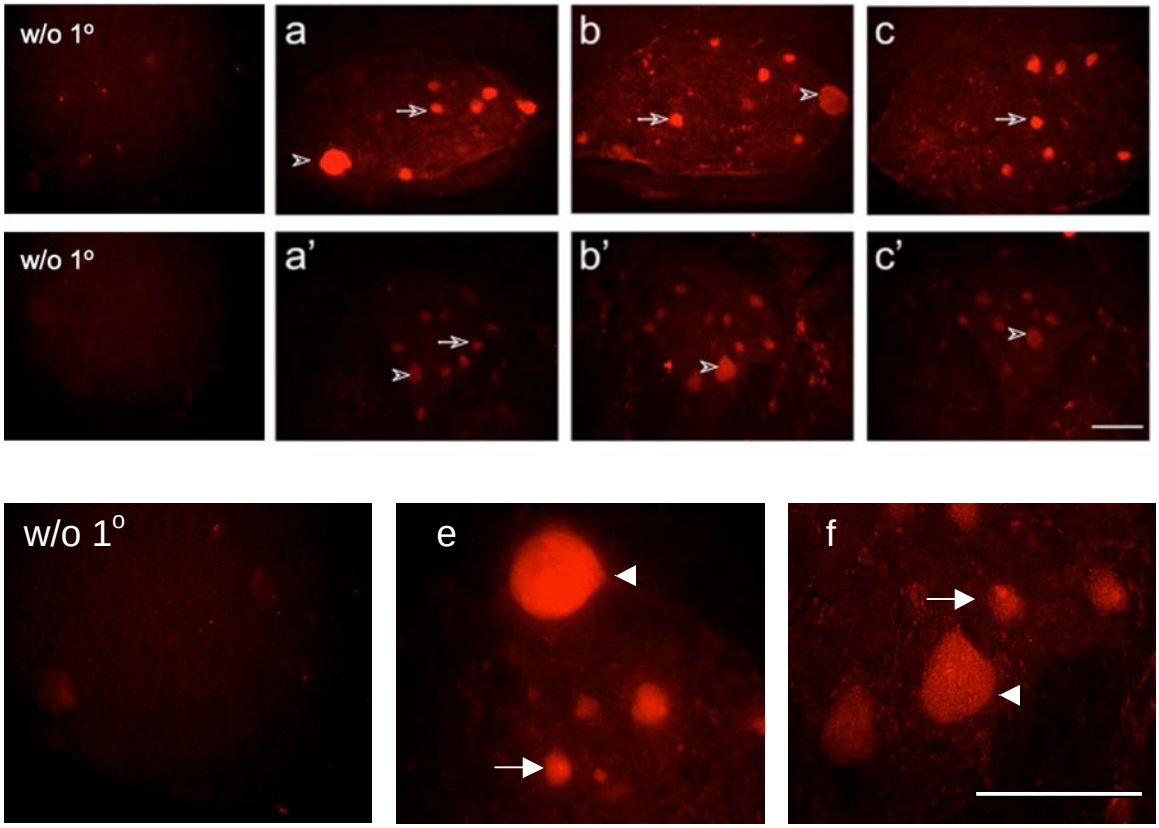


Figure 26: Calcineurin (PP2B) distribution in the PMn of *A. leptorhynchus* and *E. virescens.*

Serial sections through PMn of *A. leptorhynchus* and *E. virescens* were incubated with a primary antibody against PP2B. Calcineurin expression in *A. leptorhynchus* (Panels a, b and c; 20X magnification; n=3) and *E. virescens* (Panels a', b' and c'; 20X magnification; n=3). Calcineurin expression was observed in both pacemaker (arrows) and relay neurons (arrowheads) in both species. (e and f). Increased magnification (40X) of *Apteronotus* and *Eigenmannia* calcineurin expression, respectively. Control experiments were performed without primary antibody (w/o 1°). Scale Bar = 100 microns.

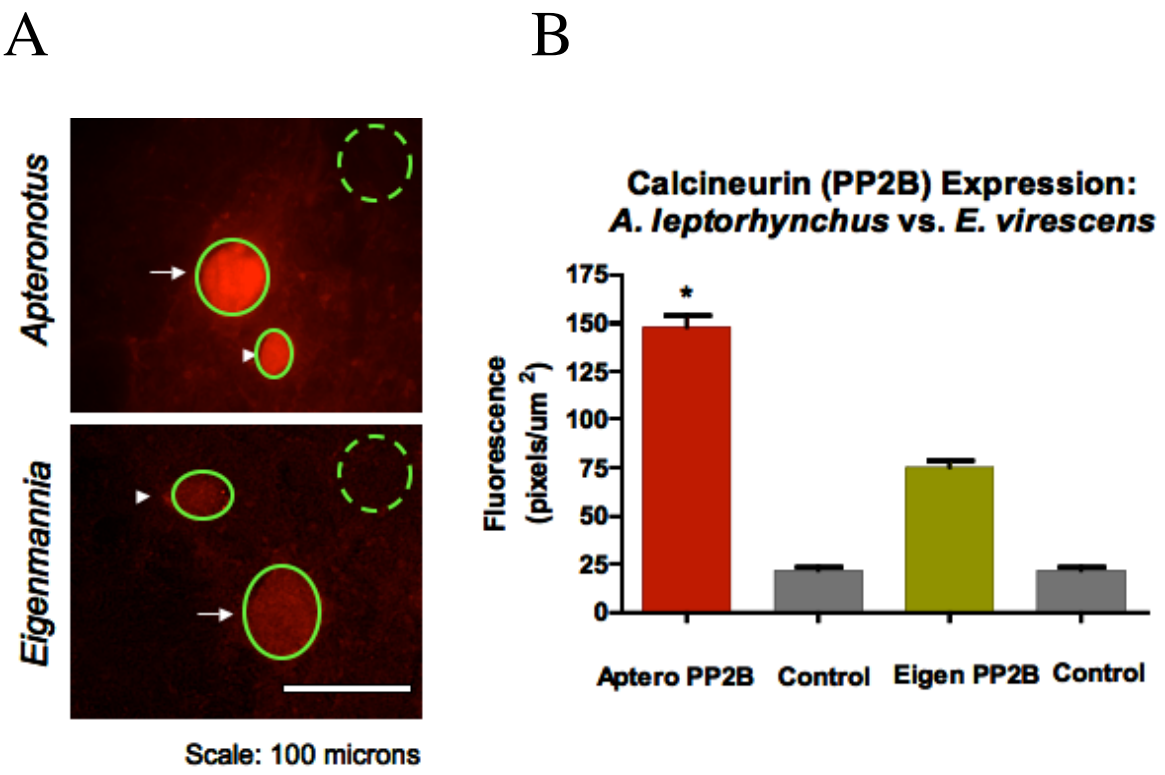


Figure 27: Quantitative differences in calcineurin (PP2B) expression.

(A) Serial sections through pacemaker nuclei of *A. leptorhynchus* and *E. virescens* were incubated with or without a primary antibody and PP2B immunofluorescence was quantified by averaging fluorescence intensities from pacemaker and relay neurons (solid green circles) and measured against unlabeled regions (dotted green circles). (B) Group data form PP2B immunohistochemistry showed differences in fluorescence intensities (measured as pixels/um^2) when compared to regions showing little or no PP2B expression ($p < 0.05$). Data analysis performed by paired student's T-test.

DISCUSSION

These findings demonstrate that in the weakly electric fish, *A. leptorhynchus* and *E. virescens*, the pacemaker nuclei of these two species (PMn) differentially express conventional and atypical PKCs. In *A. leptorhynchus* PKCα is abundant. Conversely, PKCα is nearly absent in the PMn of *E. virescens*. PKCβ isoform is equally expressed in the PMn of both species while PKCγ, an isoform solely expressed in the brain and spinal cord and its localization is restricted to neurons is absent. PKCζ, an atypical isoform is abundantly expressed in the PMn of both species. In addition, we demonstrate that calcineurin (a calmodulin-dependent protein phosphatase) expression in *A. leptorhynchus* is twice that of *E. virescens*. Together, these data may explain the neural mechanisms that contribute to the species-specific differences in LTFE.

Differential Expression of Conventional PKCs in *A. leptorhynchus* and *E. virescens*

Calcium-induced protein phosphorylation can alter the functional state of various proteins (Lieberman and Mody, 1994; Pingle et al., 2007; Scholz and Palfrey, 1998; Shabir et al., 2004; Shistik et al., 1999). Recently, activity-dependent protein phosphorylation has provided insight into synaptic plasticity through the regulation of proteins in the postsynaptic density (PSD), specifically, cytoskeletal and scaffolding proteins, receptors, ion channels and signaling molecules (Hou et al., 2005; Lin et al., 2006; Shen and Meyer, 1999; Trinidad et al., 2005). The phosphorylation state of components of the PSD is central to synaptic transmission and is known to play a role in synaptic plasticity and provides a rationale framework to understand the how plasticity can occur within the central nervous system.

In the present study, western blotting analysis demonstrates that the brain of *A. leptorhynchus* expresses proteins with similar molecular masses of conventional PKC isoforms PKCα and PKCβ. These isoforms are differentially expressed and can be found in a wide variety of tissues (Buchner et al., 1999; Clarke et al., 2000; Ford et al., 1995; Pinton et al., 2002) with only two known isoforms (PKCγ and PKCζ) known to be expressed solely in the CNS (Sacktor, 2008; Shearman et al., 1989). In *A. leptorhynchus*, the conventional isoforms PKCα and PKCβ are expressed in the pacemaker nucleus and is consistent with previous findings that conventional PKCs are expressed in the *Apteronotid* central nervous system (Maler, 1999). Interestingly, PKCγ is expressed in the brain of in *A. leptorhynchus* but is absent in the PMn indicating that PKCγ is not responsible in regulating either PMn spontaneous activity or LTFE. If the extent of involvement of each enzyme relates to its abundance, then *A. leptorhynchus* regulates LTFE with both PKCα and to a lesser extent PKCβ (**Figure 24**).

In the mammalian cerebellar cortex, PKCα, PKCβ are found in Purkinje cells, and PKCβ is expressed in climbing fibers, and granule cells/parallel fibers (Garcia and Harlan, 1997). In mammals, PKC is required for induction of hippocampal LTP (Malinow et al., 1989) and LTD in cerebellar Purkinje cells (De Zeeuw et al., 1998; Linden and Connor, 1991). It will be of important interest to determine whether PKCs are differentially expressed in pacemaker and relay neurons, the two primary cell types that contribute to PMn spontaneous activity.

In the present study, Western blotting revealed that PKCα and PKCγ are expressed in total brain of *E. virescens* but are absent in the PMn. In addition, these findings demonstrate that *E. virescens* does not primarily rely on conventional isoforms to regulate PMn activity and suggests that an alternative kinase is involved in neuronal plasticity or basal firing rate of the PMn. Calcium/calmodulin-dependent kinase 2

(CaMKII) is a multifunctional serine/threonine kinase activated by calcium/ calmodulin; it is abundant in the nervous system where it is highly enriched at synaptic sites (Lisman et al., 1997). Recently, mapping of the distribution of both isoforms of CaMKII in the brain of *Apteronotus leptorhynchus* has demonstrated expression in the lateral line lobe (ELL) where this enzyme may be involved in plasticity at both pre- and postsynaptic sites (Wang and Maler, 1998). Future investigation into the expression of CaMKII as alternative calcium-mediated kinase in the regulation of PMn activity in *A. leptorhynchus* and *E. virescens* will determine if CaMKII is differentially expressed in the PMn two species and potentially be an additional kinase needed for the regulation of PMn plasticity.

Expression of Atypical PKCs in *A. leptorhynchus* and *E. virescens*

Previously studies have found that protein the kinase Mzeta (PKMzeta), an autonomously active form of the atypical PKCζ isozyme increases at synapses during long-term potentiation (LTP) and has been shown to enhance excitatory synaptic transmission by increasing the number of active postsynaptic AMPA receptors (Ling et al., 2006; Sacktor et al., 1993). In total brain and PMn extracts, using an antibody specific to the catalytic domain of PKCζ western blotting revealed bands corresponding to a single protein ~80KDa for both *Apteronotus* and *Eigenmannia*.

As described in the previous chapter, PKCα, PKCβ and PKCζ are constitutively active in the PMn of *A. leptorhynchus* (see chapter 2). At this time it is unknown whether the catalytic fragments are translated *de novo* and regulate PMn spontaneous activity or if all three kinases are coupled to their regulatory domains and, in turn, undergo cleavage by enzymes (Bougie et al., 2009; Villareal et al., 2009). PKMz can be transcribed from

an internal promoter within the PKCζ gene and future experiments will determine whether this level of regulation occurs in one or in both species.

PMn Calcineurin (PP2B) expression in *A. leptorhynchus* and *E. virescens*

In contrast to the large number of protein kinases that have been discovered and studied in detail, relatively few protein phosphatases have received similar attention although, several have been implicated in mammalian models of learning and memory (Misonou et al., 2004; Mohapatra et al., 2009; Park et al., 2006). Type II phosphatases are subdivided into those that are spontaneously active (PP1 and PP2A), Ca^{2+}/dependent (PP2B), and Mg^{2+}-dependent (PP2C) classes of phosphatases (Belmeguenai and Hansel, 2005). Western blotting revealed a protein corresponding to ~ 61 KDa in both total and PMn extracts from *Apteronotus* and *Eigenmannia*. However, immunoblotting revealed a band with slightly higher molecular weight in *Apteronotus*.

This difference in molecular weight could be explained by the expression of differential expression of calcineurin isoforms or, perhaps, calcineurin is phosphorylated in *Apteronotus* and not in *Eigenmannia*. To test the latter, we incubated PMn extracts from *Apteronotus* with Antarctic phosphatase. Immunoblotting revealed a shift in the band corresponding to ~61 KDa from the higher molecular weight product demonstrating that calcineurin is phosphorylated in the PMn of *Apteronotus* (data not shown).

If calcineurin acts as an inhibitory constraint on PMn excitability by rapidly dephosphorylating target proteins involved LTFE (see chapter 3), is it possible that the species specific differences in the duration of LTFE are truly regulated by a single signaling cascade through the activation of calcineurin? In *Apteronotus*, *in vivo* experiments have demonstrated that with a strong jamming stimulus, LTFE duration lasts ~ 2 hours. However, in the shoaling species, *Eigenmannia*, the same stimulus produces a

LTFE lasting as long as 16 hours (Oestreich and Zakon, 2005). If the amount of calcineurin expression correlates with the duration of LTFE (i.e. the more calcineurin expressed the shorter the duration of LTFE), then this could potentially explain the observed differences in species-specific neural plasticity. In hippocampal CA1 pyramidal neurons activation of glutamate receptor and increases in $[Ca^{2+}]i$ can also lead to dephosphorylation, altered translocation and a shift in the voltage dependent activation of Kv2.1 channels whereby calcineurin (PP2B) activation is both necessary and sufficient for these effects (Misonou et al., 2004; Mohapatra et al., 2009; Park et al., 2006). More importantly, these data demonstrate that calcineurin regulates mechanisms of neural plasticity and does not contribute to the basal electrical activity of these cells.

Using serial sections from the brains of *Apteronotus* and *Eigenmannia* were incubated with a primary antibody against calcineurin to identify specific cell types that express calcineurin. Immunolabeling revealed calcineurin expression in both pacemaker and relay neurons in both species. However, quantifying the average immunofluorescence in both species revealed a two-fold increase in the amount of calcineurin expression in the PMn of *Apteronotus* compared to *Eigenmannia*. Together, these data demonstrate that differences in PKC and calcineurin expression mirror differences in social structure and future experiments will determine whether PMn PKCs contribute to LTFE in *E. virescens*.

Chapter 5: Conclusion

This dissertation primarily revolved around the question of whether calcium, primarily working through enzymatic processes, contributes to the regulation of PMn spontaneous activity as well as long-term changes in pacemaker excitability. I showed that conventional and atypical PKCs contribute to the regulation of PMn spontaneous activity as well as long-term changes in pacemaker excitability while alternative mechanisms, such as calcineurin, are recruited only during synaptically-induced plasticity.

I showed that that a novel form of synaptically induced neural plasticity, long-term frequency elevation (LTFE) in the pacemaker nucleus of *Apteronotus leptorhynchus,* is regulated by conventional (PKCα and PKCβ) and atypical (PKCζ) PKCs. I also showed that Ca2+/calmodulin-dependent kinase II (CaMKII), an effector that has been shown in several studies to regulate neuronal plasticity, does not play a role in PMn basal firing rate or LTFE. In addition, I demonstrated that calmodulin and the calmodulin-activated phosphatase, calcineurin, are recruited only during synaptically induced changes in PMn excitability and regulate the maintenance of LTFE. Together with the PMn calcium imaging data, I showed that these effectors work downstream of NMDAR activation. In addition to the mechanisms that regulate LTFE, I also demonstrated that conventional and atypical PKCs interact non-additively ensure remarkable fidelity in the output of this neural circuit.

Further, I demonstrated that the weakly electric fish, *A. leptorhynchus* and *E. virescens*, differentially express conventional and atypical PKCs. In the course of these experiments, I showed that *A. leptorhynchus* expression of PKCα in the PMn is abundant whereas in *E. virescens*, the isoform is nearly absent. Pacemaker expression of PKCβ

nearly equal both species while PKCγ, is absent. Equally, I demonstrated that expression of PKCζ, is abundantly expressed in the PMn of both species. In addition, I demonstrated that Ca^{2+}/calmodulin dependent phosphatase, calcineurin expression in *A. leptorhynchus* is greater than *E. virescens*.

In the following discussion I discuss how long-term changes in neuronal intrinsic excitability in the electromotor system could be instructive in studying the cellular basis for changes in intrinsic membrane properties and how it relates to learning and memory. Finally, I will discuss the problems I encountered in my research, and about potential future directions for the project.

Using Rhythmic Circuits in Studying Neuronal Homeostasis and Activity-Dependent Changes in Intrinsic Excitability

Studying homeostasis and plasticity by using spontaneously active neuronal circuits

Neurons can display a variety of activity patterns that depend on the number and type of voltage channels in their membranes. Some neurons are silent unless excited; others are spontaneously active. Some neurons display intrinsic oscillatory properties involving periodic bursts of action potentials (Marder et al., 1995). Neuronal activity itself modifies not only synaptic efficacy but also the intrinsic membrane properties of neurons. Several studies suggest that memory in networks results from interaction between changes in synaptic efficacy and intrinsic membrane properties (Marder et al., 1996; Marder and Calabrese, 1996; Skinner et al., 1994; Turrigiano et al., 1996).

Spontaneously active neurons possess intrinsic currents that interact to depolarize the cell membrane to threshold and repolarize the membrane to negative potentials from which the action potential can be initiated. Several studies have identified currents responsible for the subthreshold depolarizations leading to each spike. The

depolarizations can result from dynamic recruitment and activation of hyperpolarization-activated channels and T-type Ca^{2+}channels (McCormick and Huguenard, 1992). In other neurons, voltage-gated Na^{+} currents and/or nonselective cation currents bring the membrane potential to threshold (Do and Bean, 2003; Jackson et al., 2004; Raman et al., 2000).

Spontaneous firing is not only a prominent feature of many neuronal networks but may also serve useful functional roles, contributing to regulating information flow in different microcircuits in the brain (Hausser et al., 2004). Spontaneous firing plays a central role in transforming synaptic input into spike output and encoding plasticity in a wide variety of neural circuits (Feigenspan et al., 1998; Hausser and Roth, 1997; McCormick and Pape, 1990; Raman and Bean, 1997; Smith and Otis, 2003; Taddese and Bean, 2002). The advantage of studying circuits that exhibit spontaneous activity is that it provides an investigator with functional results that are readily interpretable.

The pacemaker nucleus of the *Apteronotus* is a prime example of a system that can be exploited to measure, in real-time, neuronal stability and activity dependent changes in excitability. Representing one of the most stable neural circuits, the PMn firing rate oscillates with submicrosecond precision and can be used to study mechanisms of learning from the cellular mechanisms that modulate short and long-term neuronal responses to system wide, stable changes that contribute to memory formation. However, LTFE differs from existing theories for learning and memory in that it develops from a single graded stimulus presentation and can be described as a nonassociative memory process, because it is activated by a single modality, a pure sine wave within a few Hertz of the fish's EOD frequency.

Resetting of PMn output represents a form of sensorimotor adaptation

Vertebrates process sensory information in a multimodal fashion with the objective of performing specific tasks in any given environment. Sensorimotor adaptations arise from long-term changes in motor output as a consequence of long-term changes in sensory input. Adaptation to the spatial distortions introduced by prisms is a well-known phenomenon (Podell et al., 1982; Redding et al., 1992). A subject wearing wedge prisms experiences compressions, extensions, and distortions of the visual scene that depend on saccadic eye movements and head movements. With time, subjects adapt to these unusual correlations between movements and sensory changes. As a consequence, when the prisms are removed, aftereffects are obtained that can be measured by the strength of prisms, oriented in the opposite direction, necessary to cancel the perceptual distortion experienced when eye or head movements are performed.

Central to these changes and adaptations are neural events underlying vestibular function, vision, proprioception, and the integration of sensorimotor function. In the weightless environment of space, the vestibular otolithic receptors and the tactile proprioceptors no longer signal changes in body orientation as they do on earth. Central motor programs for the reinterpretation of sensory inputs and coordination of muscle actions must undergo adaptation. It is hypothesized that the rearrangement and mismatch of sensory cues gives rise to the syndrome of space motion sickness, to which the body gradually adapts (Edgerton et al., 2001).

Both of these examples represent perturbations that occur in sensory modalities that corrected to produce stable changes in motor output. LTFE represents a form of sensorimotor adaptation whereby an error signal (in the form of the beat frequency generated by the signal interaction of two fish with similar EODs) is sensed by the fish and the recalibration of PMn firing rate is adaptively altered to avoid the jamming of their

electrosensory perception. Electric fish in their natural environment often interact for long periods and LTFE represents a physiological adaptation that allows an individual fish the luxury of maintaining effective electrolocation and communication without constantly moving in and out of different local environments, which in turn, reduces the chance of predation.

The underlying mechanisms of LTFE

The quantitative correlation between synaptic strength and LTFE magnitude could be based on the amount of Ca^{2+} influx through NMDA receptors into pacemaker and/or relay neurons. It is possible that changes in intracellular Ca^{2+} concentrations or averaging spike rate would measure the duration and strength of exposure to a signal intruding on the sensory space and adjust their frequency shift appropriately to avoid jamming.

Previous studies have focused on a class of ion channels known as TRP channels that may underlie the maintenance of LTFE (Oestreich et al., 2006). These Ca2+ activated nonspecific cation currents (CAN currents) have been described in a number of neural systems (Partridge et al., 1994). CAN currents do not inactivate and can produce sustained depolarizations, and therefore could cause the increase in intrinsic excitability observed in the electromotor pacemaker nucleus. Ca^{2+}-activated alterations of these currents has been described to produce a graded memory in entorhinal cortex neurons in vitro and are expressed in hippocampal CA1 neurons as well as the *Apternotid* PMn (Egorov et al., 2002, Partridge and Valenzuela, 2000).

However, the findings in this treatise support the hypothesis that the influx of Ca^{2+}- through NMDARs initiates a signaling cascade that involves Ca^{2+}- dependent protein phosphorylation. At this time it is unclear whether PMN TRP channels are modulated by PKC and/or calcineurin. It is tempting to speculate that one possible target of these effectors are TRP channels. However, we cannot rule out the possibility that the ionic currents that contribute to PMn autonomous activity are targets themselves. As stated previously (see chapter 3) PMn spontaneous activity is regulated by at least three ionic currents regulate the firing frequency of neurons in the brown ghost PMn: (1) a sodium current that is sensitive to TTX and μOCTX MrVIA; (2) a potassium current that is sensitive to 4AP and κACTX SIVA, but resistant to cesium, TEA, αDTX, and AGI-2; and (3) a calcium current that is sensitive to nickel and cadmium, but resistant to specific blockers of L-, N-, P-, and Q-type calcium currents.

Cellular mechanisms of neuronal intrinsic excitability

Many different neurons have the capacity to change their intrinsic membrane properties in response to different stimuli. Studies in cortical and hippocampal pyramidal neurons have shown the importance of active electrical processing of synaptic input by somatodendritic voltage-dependent ion channels. Neuronal excitability is regulated by ionic conductances that may be targets for the cellular changes that support learning and memory (Nelson et. al., 2003).

Several studies demonstrate that kinase activity can be linked to changes in pyramidal cell intrinsic excitability. PKC activation by phorbol esters (e.g. PMA or PBDu) can decrease the afterhyperpolarization (I_{AHP}) and abolishing spike frequency adaptation after electrical stimulation (Malenka et al., 1986; Seroussi et al., 2002). In neurons of the deep cerebellar nuclei (DCN) associative eye blink conditioning is

accompanied by a sustained increase in firing rate as well as an increase in the acquisition of the conditioned response (Aizeman and Linden, 2000). The plasticity of excitability in neurons of the medial vestibular nucleus (MNV), a brainstem nucleus essential for motor learning in the vestibulo-ocular reflex (VOR), synaptic inhibition or membrane hyperpolarization evoke firing rate potentiation via reductions in BK-type calcium-activated potassium currents (Nelson et al., 2003). Because these neurons are spontaneously active, local concentrations of calcium are maintained leading to the constitutive activity of CaMKII and recent findings demonstrate that inhibition of CaMKII leads to an increase in excitability by reducing BK-type currents (Nelson et al., 2005).

The neurons of the PMn are unique in that they utilize conventional and atypical PKC isoforms to set the PMn basal firing rate in a cooperative, possibly serial fashion and exploit these same kinases for LTFE. This interaction represents the first known example that the cooperation between PKCs can regulate neuronal stability (manifest as the constancy in PMn firing rate).

Limitations of this Dissertation Project

The resolution of PMn calcium and the induction of LTFE

Imaging PMn Ca^{2+} transients during inhibition of PKC and calcineurin demonstrated that relative cytosolic calcium levels are not altered when matching LTFE induction. However, PMn Ca^{2+} transients were sampled every 2 seconds and do not represent the actual levels of Ca^{2+} during stimulation. We define the induction of LTFE as an arbitrary time point (40 seconds after stimulation) outside of what is considered the actual induction of the frequency elevation. Therefore, the ratiometric values are an

indication that the mechanisms that regulate LTFE induction have occurred intact. A true measure of PMn Ca^{2+} would require an increase in sampling resolution during electrical stimulation.

Making the connection between *A. leptorhynchus* and *E. virescens*

The immediate concern facing this line of investigation is whether or not the same processes that contribute to the regulation of PMn spontaneous firing and changes in PMn plasticity are the same as or different from those in the shoaling gymnotifom *Eigenmannia virescnes*. Although these experiments have identified differences in the expression of PKCs and calcineurin, their functional significance in *Eigenmannia* is unknown. The main difficulty we face in answering this question is that the PMn of *Eigenmannia* remains experimentally inaccessible.

In *E. virescens*, the PMn nuclei lies deep within the brainstem and receives tonic input from the sublemniscal prepacemaker nucleus (SPPn). Several attempts have been made to isolate the PMn *in vitro*. Consequently, the preparation fails to autodepolarize *in vitro* and is likely due to uncoupling of the nucleus by severing the connections from the SPPn to the nucleus. The PKC expression assays are circumstantial and until a viable *in vitro* slice preparation is established in *Eigenmannia*, assessing the role of Ca^{2+} dependent phosphorylation/dephosphorylation will be challenging.

FUTURE STUDIES

The electromotor system of weakly electric fish is a remarkable model system that allows investigation into several phenomena that underlie long-term adaptive changes in motor output. However, a number of experiments are needed to support this current line

of investigation and provide a foundation for further investigation into the mechanisms that underlie neuronal stability and the mechanisms that govern plasticity.

Identifying the molecular targets that contribute to LTFE

Initially, the identifications of phosphoproteins that lie downstream of NMDAR activation could be assayed by immunoprecipitating the effectors identified in this dissertation to identify their coupling with other proteins. Primary antibodies against PKCs and calcineurin would be used to pull-down complexes before and after the induction of LTFE to identify, initially, the proteins coupled to these effectors and, subsequently, if the state of phosporhylation of those proteins were altered by either direct NMDAR activation or electrical stimulation of the SPPn.

Identifying the ionic currents underlying LTFE

The best approach for identifying currents that underlie LTFE is whole-cell voltage clamp of PMn neurons. However, several attempts to isolate currents by our lab and other labs (Troy Smith and Ray Turner, personal communication) have been unsuccessful. Several attempts to dissociate PMn neurons also have been unsuccessful due to various technical and physiological difficulties. As stated previously, PMn basal firing rate is regulated by 4 identified ionic currents that could serve as potential mechanisms, which in addition to regulating spontaneous activity could regulate LTFE.

Investigating long-lasting calcium transients of the PMn

As described in chapter 3, PMn calcium transients often last on the order of 5 minutes after stimulation of the SPPn pathway. Given this extremely long calcium signal, how do these neurons handle such a long-lasting calcium load without being neurotoxic?

Equally, what are the underlying calcium buffering mechanisms that keep PMn neurons form reaching toxic levels? Unlike other weakly electric fish, the electric organ of *Apteronotus* continues to discharge for -24 hr after the spinal cord is transected (Dye and Meyer, 1986; Schaefer and Zakon, 1996) suggesting that spinal neurons in the electromotor circuit also fire spontaneously (like pacemaker cells) at an intrinsic rate. The electromotor neurons express of the spinal cord highly express calbindin-D28k, a member of a large family of intracellular calcium-binding proteins containing EF-hand calcium binding motifs and related to calmodulin and troponin-C. The high expression of calbindin could potentially serve as one mechanism that could buffer calcium in pacemaker and/or relay neurons.

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Vita

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